Unwavering friendship

Exploring insect-microbe symbiosis in *Nezara viridula*

Magda A. Rogowska-van der Molen

Radboud Institute for Biological and Environmental Sciences

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Exploring insect-microbe symbiosis in *Nezara viridula*

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door

Magda Aleksandra Rogowska-van der Molen
geboren op 6 september 1994
te Olsztyn (Polen)

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Dr. A. Berasategui Lopez (Vrije Universiteit Amsterdam)

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Dissertation to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.M. Sanders,
according to the decision of the Doctorate Board
to be defended in public on
Wednesday, July 3, 2024
at 12:30 pm

by

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born on September 6, 1994
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To my children

Summary

Throughout evolution, insects have developed exceptional adaptive capabilities by forming symbiotic relationships with microbes, which are often crucial for their survival. Among shield bugs, such as the Southern green shield bug *Nezara viridula*, mutualistic interactions with bacteria are common and contribute significantly to the insect's ecological success. *N. viridula* is a piercing and sucking insect that relies on symbionts for nutrient supplementation. It is a highly invasive species with a broad dietary range across many plant families and its global spread results in significant agricultural losses. Recent studies showed that the shield bug microbiota can manipulate plant defences, affecting pest insect damage potential, however, the role of *N. viridula* microbiota remains unexplored.

In this PhD thesis, we address knowledge gaps in the insect-microbe symbiosis field and aim to understand the role and relationship between *N. viridula* and its associated microbes. In **Chapter 1** a general introduction to the topic of the thesis is provided. **Chapter 2** reviews the current literature on microbial degradation pathways of toxic secondary plant metabolites. Plants produce a variety of secondary metabolites in response to biotic and abiotic stresses, including alkaloids, glucosinolates, terpenes, and polyphenols. Microbes present in diverse ecological niches have been found capable of detoxifying these metabolites, some of which are associated with insects through detoxifying symbiosis.

In **Chapter 3**, the function of the *N. viridula*-associated microbial community was investigated through inference from metagenome-assembled genomes, 16S rRNA gene amplicon sequencing, isolated strains and plant infection assays. We identified the core bacterial microbiota across the developmental stages of *N. viridula* and determined that *Pantoea* and *Sodalis* are vertically transmitted. Our findings showed that *N. viridula* microbiota have the potential to supplement nutrients and participate in digestion. Moreover, we demonstrated that *Serratia* symbionts detoxified 3-nitropropionic acid *in vitro* and that insect-associated microbes repressed plant defences directed towards insects.

In **Chapter 4**, we took a closer look at the *Sodalis* symbiont of *N. viridula*. We determined that *Sodalis* colonizes salivary glands, anterior regions of the midgut and testes. Surface sterilization of *N. viridula* eggs resulted in a decreased survival rate and fitness of the insect, however, it did not disrupt the acquisition

of *Sodalis* symbionts, which were possibly stored inside eggs. Genomic analysis of *S. nezarae* indicated that the genome displayed traits of instability typical of endosymbiotic lineages, which suggests ongoing speciation from a non-host-associated ancestor to an obligate symbiont. Since we have not observed any microbes associated with ovaries but found *Sodalis* in testes, we propose that male shield bugs might be involved in the vertical transmission of symbionts.

Chapter 5 focused on the comparative genomic analysis of the facultative *Commensalibacter* sp. Nvir symbiont with other insect-associated species of that genus. We observed that *Commensalibacter* possesses a reduced genome, yet harbours the largest number of unique genes related to carbohydrate metabolism. Additionally, *Commensalibacter* demonstrated the capability to biosynthesize amino acids and B vitamins, potentially benefiting the host. The genome also displayed adaptation to a symbiotic lifestyle and the ability to detoxify plant toxins.

In **Chapter 6**, we further investigated the participation of *N. viridula* microbes in detoxifying symbiosis and determined factors that affect gut microbial community composition. We found that core- and transient-associated gut microbes detoxified 3-nitropropionic acid *in vitro* and determined that the toxin might inhibit amino acid supplementation to the host. Testing pairwise interactions between the isolated microbial members revealed that core members support each other and repress the growth of transient microorganisms. In line with these findings, we found that switching to a single-plant diet shifts the abundance of core microbes.

Chapter 7 delved into the characterization of the 3-nitropropionic acid degradation pathway by *Pseudomonas* sp. Nvir isolated from the gut of *N. viridula*. We determined that the strain used the toxin as carbon, nitrogen and energy source as it was able to grow on it *in vitro*. Furthermore, using transcriptomics we identified the genes involved in the breakdown pathway and discovered a novel putative nitronate monooxygenase gene, whose corresponding enzyme was probably involved in the first step of toxin degradation. The phylogenetic analysis of nitronate monooxygenase revealed the presence of the gene in many bacterial families, suggesting a widespread occurrence of 3-nitropropionic acid detoxification ability among microbes.

Finally, **Chapter 8** offered a thorough synthesis of the thesis findings by integrating research outcomes. It delved into the potential implications of

the acquired knowledge in insect-plant-microbe interactions in exploring unculturable bacteria and pest management strategies. The chapter provided a critical analysis of the conducted experiments and concluded by proposing suggestions for future research.

In summary, this PhD thesis has broadened our understanding of insect-microbe symbiosis in *N. viridula* with a special focus on elucidating the role of microbial community members and their relationship with the host. This study laid a solid foundation for future research in the area of insect-microbe interactions and their application for agricultural practices and pest management.

Samenvatting

Door de evolutie heen hebben insecten een uitzonderlijk aanpassingsvermogen ontwikkeld door symbiotische relaties aan te gaan met microben die vaak cruciaal zijn voor hun overleving. Onder schildwantsen, zoals de zuidelijke groene schildwants, komen mutualistische (met wederzijns voordeel) interacties met bacteriën vaak voor en dragen ze sterk bij aan het ecologische succes van dit insect. *N. viridula* is een plant-doorborend en opzuigend insect dat afhankelijk is van symbionten voor de supplementatie van voedingsstoffen. Het is een zeer invasieve soort met een brede voedingskeuze in veel plantenfamilies, waarvan de wereldwijde verspreiding leidt tot aanzienlijke landbouwverliezen. Recente onderzoeken hebben aangetoond dat de microbiota van de schildwants de afweer van planten kan manipuleren, wat een effect kan hebben op de schadelijkheid van de insecten. De rol van de microbiota van *N. viridula* is echter grotendeels onbekend.

In dit proefschrift richten we ons op kennislacunes op het gebied van symbiose tussen insecten en microben en streven we ernaar de rol en relatie tussen *N. viridula* en de daarmee geassocieerde microben te begrijpen. In **Hoofdstuk 1** wordt een algemene inleiding op het onderwerp van het proefschrift gegeven. **Hoofdstuk 2** geeft een overzicht van de huidige literatuur over microbiële afbraakroutes van toxische secundaire plantmetabolieten. Planten produceren een grote verscheidenheid aan secundaire metabolieten als reactie op biotische en abiotische stress, waaronder alkaloïden, glucosinolaten, terpenen en polyfenolen. Microben uit diverse ecologische niches zijn in staat gebleken deze metabolieten te ontgiften en een aantal van deze microben is geassocieerd met ontgiftende symbiose met insecten.

In **Hoofdstuk 3** werd de functie van de *N. viridula*-geassocieerde microbiële gemeenschap onderzocht door middel van metagenoom-geassembleerde genomen, 16S rRNA gen amplicon sequencing, geïsoleerde stammen en plantinfectietesten. We identificeerden de belangrijkste bacteriële microbiota in de ontwikkelingsstadia van *N. viridula* en stelden vast dat *Pantoea* en *Sodalis* verticaal worden overgedragen. Onze bevindingen toonden aan dat *N. viridula* microbiota het potentieel heeft om voedingsstoffen te supplementeren en deel te nemen aan de spijsvertering. Bovendien hebben we aangetoond dat *Serratia*-symbionten *in vitro* 3-nitropropionzuur ontgiften en dat insect-geassocieerde microben de afweer van planten tegen insecten onderdrukten.

In **Hoofdstuk 4** hebben we de *Sodalis*-symbiont van *N. viridula* verder onderzocht. We hebben vastgesteld dat *Sodalis* de speekselklieren, de voorste delen van de middendarm en de testikels koloniseert. Oppervlaktesterilisatie van *N. viridula*-eieren resulteerde in een verminderde overlevingskans en fitheid van het insect, maar het verstoorde de overdracht van *Sodalis*-symbionten, die mogelijk in eieren werden opgeslagen, niet. Genomische analyse van *S. nezarae* gaf aan dat het genoom kenmerken van instabiliteit vertoonde die typerend zijn voor endosymbiotische afstammingen, wat duidt op voortdurende soortvorming van een niet-gastheer-geassocieerde voorouder naar een obligate symbiont. Omdat we geen microben hebben waargenomen die geassocieerd zijn met eierstokken, maar we wel *Sodalis* in de testikels hebben gevonden, stellen we voor dat mannelijke schildwantsen mogelijk betrokken zijn bij de verticale overdracht van symbionten.

Hoofdstuk 5 concentreerde zich op de vergelijkende genomische analyse van de facultatieve *Commensalibacter* sp. Nvir symbiont met andere insect-geassocieerde soorten van dat geslacht. We hebben waargenomen dat *Commensalibacter* een kleiner genoom bezit, maar toch het grootste aantal unieke genen herbergt die verband houden met het koolhydraatmetabolisme. Bovendien heeft *Commensalibacter* het vermogen om aminozuren en B-vitamines te maken, wat mogelijk de gastheer ten goede komt. Het genoom vertoonde ook aanpassing aan een symbiotische levensstijl en het vermogen om plantaardige gifstoffen te ontgiften.

In **Hoofdstuk 6** hebben we de deelname van *N. viridula*-microben aan de ontgiftende symbiose verder onderzocht en factoren bepaald die de samenstelling van de microbiële gemeenschap in de darm beïnvloeden. We ontdekten dat kern- en tijdelijk-geassocieerde darmmicroben *in vitro* 3-nitropropionzuur ontgiften en constateerden dat het toxine de aminozuursuppletie aan de gastheer zou kunnen remmen. Het testen van paarsgewijze interacties tussen de geïsoleerde darmmicroben liet zien dat kernleden elkaar ondersteunen en de groei van tijdelijke micro-organismen onderdrukken. In overeenstemming met deze bevindingen hebben we ontdekt dat het overstappen op een dieet met één plant de hoeveelheid kernmicroben beïnvloedt.

Hoofdstuk 7 verdiepte zich in de karakterisering van de afbraakroute van 3-nitropropionzuur door *Pseudomonas* sp. Nvir geïsoleerd uit de darmen van *N. viridula*. We stelden vast dat de stam de toxine gebruikte als koolstof-,

stikstof- en energiebron, omdat het er *in vitro* op kon groeien. Bovendien identificeerden we met behulp van transcriptomics de genen die betrokken zijn bij de afbraakroute en ontdekten we een nieuw vermoedelijk nitronaatmono-oxygenase gen, waarvan het overeenkomstige enzym waarschijnlijk betrokken was bij de eerste stap van de afbraak van toxines. De fylogenetische analyse van nitronaatmono-oxygenase onthulde de aanwezigheid van het gen in veel bacteriefamilies, wat duidt op een wijdverbreid voorkomen van het ontgiftings vermogen van 3-nitropropionzuur onder microben.

Ten slotte bood **Hoofdstuk 8** een synthese van de bevindingen van het proefschrift door onderzoeksresultaten te integreren. Het verdiepte zich in de mogelijke implicaties van de verworven kennis over interacties tussen insecten, planten en microben voor onderzoek aan niet-kweekbare bacteriën en strategieën voor ongediertebestrijding. Het hoofdstuk gaf een kritische analyse van de uitgevoerde experimenten en sloot af met suggesties voor toekomstig onderzoek.

Samenvattend heeft dit proefschrift ons begrip van de symbiose tussen insecten en microben in *N. viridula* verbreed, met een speciale focus op het ophelderen van de rol van leden van de microbiële gemeenschap en hun relatie met de gastheer. Deze studie legde een solide basis voor toekomstig onderzoek op het gebied van insect-microbe interacties en hun toepassing in de landbouw en ongediertebestrijding.

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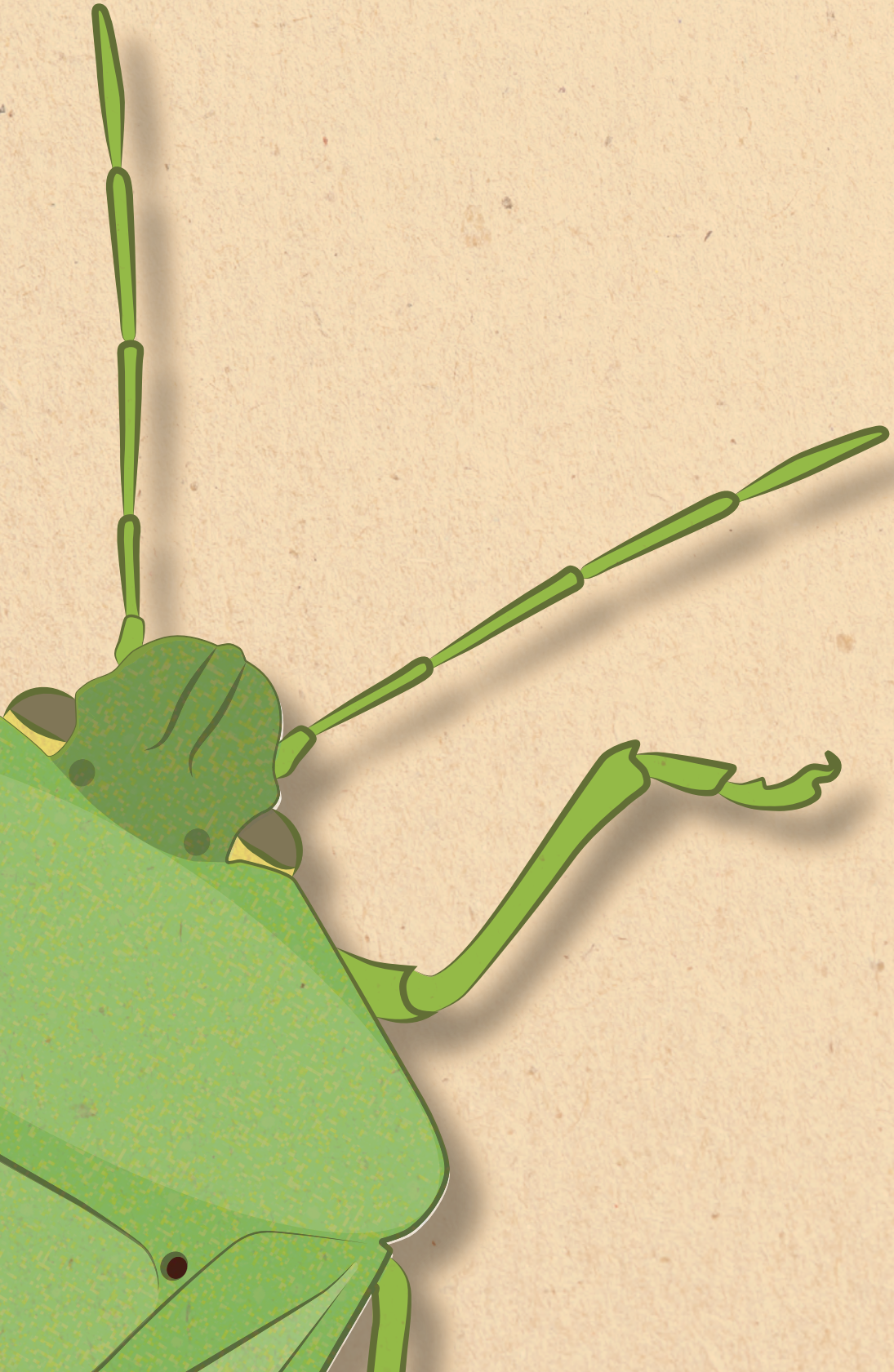

Chapter 1

Introduction

Magda A. Rogowska-van der Molen[1]

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Pest insects and global food production

Climate change is an emerging global concern that affects natural ecosystems by increasing temperatures and causing rainfalls, which endanger agriculture and global food production. The consequences of climate change can affect plant physiology and contribute to increased proliferation of pathogens, which in turn interfere with crop production. Along with that, plant resistance against diseases and pest insects is expected to become less efficient (Shrestha, 2019). The escalating temperatures associated with climate change result in increased pest populations and their damage potential (Skendzic et al., 2021). The International Centre of Insect Physiology and Ecology reported that pest insects contribute to devastating 20% of the world's total crop production annually, further emphasizing challenges in agricultural sustainability. Tackling the increasing population of pests is, therefore, crucial to meet the growing global demand for food production and requires effective measures to decrease crop losses.

While various methods such as crop rotation, chemical and biological insecticides and release of parasitoids are in use, their efficacy is often limited. Additionally, chemical insecticides have adverse effects on both human health and the environment, further adding complexity to already challenging pest control measures (Botías et al., 2019; Costa et al., 2007). For example, the unintended impact of pesticides on non-pest insects, including essential pollinators such as honey bees and bumble bees, contributes to declines in insect abundance and diversity. Considering that less than 0.5% of insects are considered pests, there is a compelling need for targeted strategies against pest insects to mitigate the effect of currently used methods on the environment (Sallam & Mejía, 2013). Integrated Pest Management (IPM) aims to achieve sustainable and economically viable pest control by integrating biological, cultural, physical, and mechanical control methods. Recent studies highlighted the potential of targeting symbiotic microorganisms as part of the IPM approach (Chung et al., 2018; Gonella & Alma, 2023). These studies indicated that insect-associated microbes play a crucial role in supporting host resistance against toxic compounds by detoxifying plant secondary metabolites and insecticides to less toxic metabolites (Figure 1.1). The impact of detoxification by insect-associated microbes on host fitness remains poorly understood, however it seems essential for the development of targeted pest control. Therefore, expanding our knowledge of how microbes help insects cope with plant

defences could lay the foundation for an alternative pest control measure, ensuring sustainable food production in the future.

Insect-associated microbes

Insects represent the largest and most diverse family of animal species. For most insects, microbes are essential and life without them severely affects their fitness and survival (Ceja-Navarro et al., 2015; Goane et al., 2022; Tada et al., 2011; W. Wang et al., 2022). Especially phloem-feeding insects whose diet is rich in carbohydrates but deficient in nutrients rely on microbial support in digestion and supplementation of essential amino acids and vitamins (Douglas, 2015). Beyond nutritional support, recent discoveries highlighted the multifaceted benefits of insect-associated microbes.

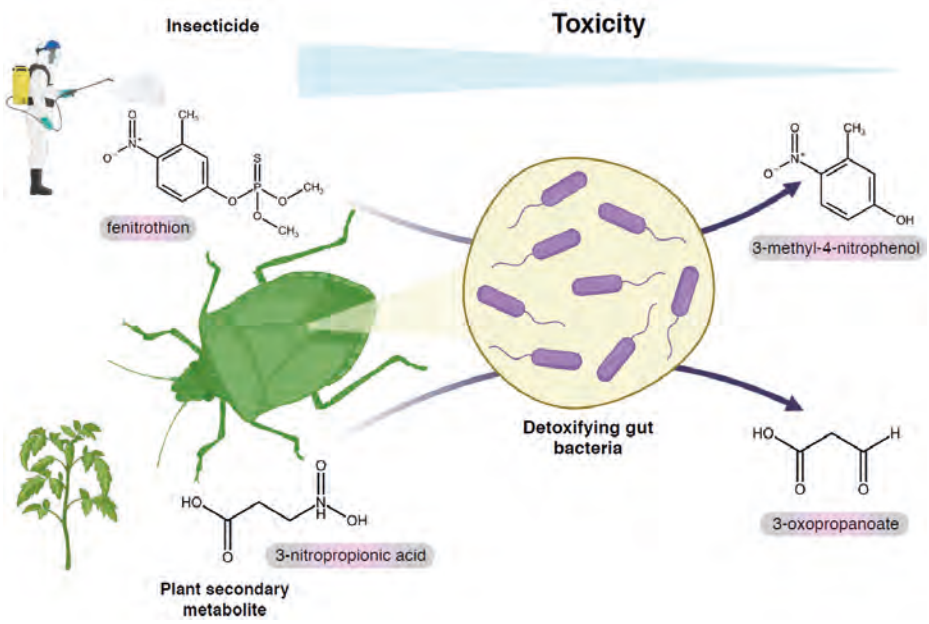

Figure 1.1. Symbiotic microorganisms degrade natural and artificial toxic compounds and confer toxin resistance to insect hosts. Created with BioRender.com.

They benefit hosts by breaking down toxic secondary plant metabolites and insecticides, endowing insects with a tremendous potential to overcome plant defences (Coolen et al., 2022; Frago et al., 2012; Harvey et al., 2003; Itoh et al., 2018; Oliveira et al., 2014; Ribeiro et al., 1994; Sato et al., 2021; Scott et al., 2021; van den Bosch & Welte, 2017). The summary of the roles of insect-associated microbes is depicted in Figure 1.2. Despite this, only a few studies explored the involvement of microbes in detoxifying symbiosis and their impact on insect performance (Berasategui et al., 2017; Pavlidi et al., 2017). Ceja-Navarro et al. (2015) and Capuzzo et al. (2005) demonstrated that the removal of gut-associated microorganisms led to retarded growth and that insects devoid of microbiota were unable to degrade plant toxins, such as caffeine and oleuropein. These findings stress the importance of symbiotic microorganisms to insect fitness and resistance against plant compounds.

Recent studies have shown that several factors determine insect microbiota composition. These include host phylogeny and dietary preferences of the host, as discussed below (Amato et al., 2019; Colman et al., 2012). Insect microbial communities, in turn, can also influence insects' host plant range, eventually affecting their pest status. Sometimes the presence of one particular species in the insect gut can substantially change the dietary preference of the host, suggesting that the pest status of insects is determined by symbiont genotype rather than insect genotype (Hosokawa et al., 2007). For example, research on pest shield bug *Megacopta punctatissima* and its close relative non-pest species *Megacopta cribraria* revealed that experimental exchange of microbiota between two species led to a significant change in the feeding pattern; the non-pest *M. cribraria* began feeding on the diet typical of pests (Hosokawa et al., 2007). As mentioned earlier, the host's diet can also shape the composition of the insect gut microbial community. A comprehensive study comparing the microbiota of 62 insect species revealed that diet strongly influenced gut bacterial community composition, suggesting that evolutionarily distinct insects may have similar microbiota (Colman et al., 2012). Moreover, pesticides and herbicides can alter the microbial community of soils and plants, promoting insect adaptation to changing environments. Therefore, understanding the interactions between insects, plants and microbes is essential in deciphering underlying detoxification mechanisms (Colman et al., 2012; Medina et al., 2018).

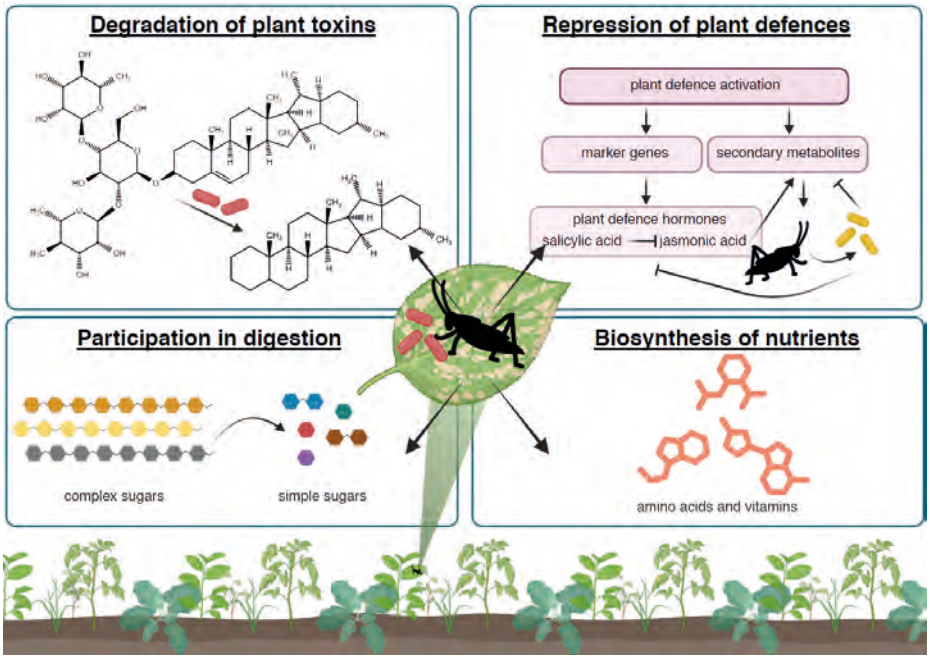

Figure 1.2. The roles of insect-associated microbes.

Pest insect *Nezara viridula*

Symbiotic associations with bacteria are frequently observed in the suborder of true bugs (Heteroptera). One such insect is the Southern green shield bug *Nezara viridula* (Pentatomidae), renowned as "one of the most important pentatomic insect pests in the world" (Todd, 1989) due to its profound ecological and agricultural impact. As a piercing and sucking insect, *N. viridula* feeds on plant phloem and xylem sap and plant cell content throughout all parts of a plant, including stems, leaf veins, growing shoots, fruits, seeds, and flowers (Meglic et al., 2001). During feeding, it employs a stylet to penetrate the tissue, injecting saliva and transmitting fungi and bacteria to plants causing the formation of leaf spots and vein necrosis (Lucini & Panizzi, 2018; Ragsdale et al., 1979). *N. viridula* is a cosmopolitan species that infests more than 30 different plant families, including Brassicaceae, Solanaceae and Fabaceae, causing damage to essential crops such as soybean, tomato, wheat, and cotton (Jones, 1988; Meglic et al., 2001).

N. viridula originates from Eastern Africa or the Mediterranean area and already in 1988, Jones (1988) predicted that its population would rapidly expand towards more Northern regions and spread globally (Hokkanen, 1986). The 2024 estimation of the Centre for Agriculture and Bioscience International showed that *N. viridula* has now been found nearly everywhere on the globe (Figure 1.3). In the Netherlands, it appears that *N. viridula* has established a local population, which was recently reported to cause damage to bell peppers, tomatoes and cucumbers in greenhouses (Aukema, 2016; van Staalduinen, 2019).

Current pest management strategies to control shield bugs primarily rely on pesticide usage. However, due to the increasing restrictions on pesticide use, more insights into *N. viridula* functioning are needed to develop alternative solutions. Recent studies showed that the shield bug microbiota can effectively manipulate and detoxify plant defences, thereby potentially influencing pest-insect host range and damage potential. This makes the insect microbiota an excellent target for the development of alternative pest control strategies (Hosokawa et al., 2007; Medina et al., 2018). The gut microbiota of *N. viridula* was shown to be involved in the detoxification and deactivation of soybean isoflavonoids and protease inhibitors *in vitro*, both known to affect insects (Medina et al., 2018). However, it remains unclear if there is an enhanced fitness effect of these microbial activities on *N. viridula.* Furthermore, additional detoxification abilities on soybean and other host plant defensive mechanisms are likely to exist within polyphagous insects such as *N. viridula* that remain to be studied.

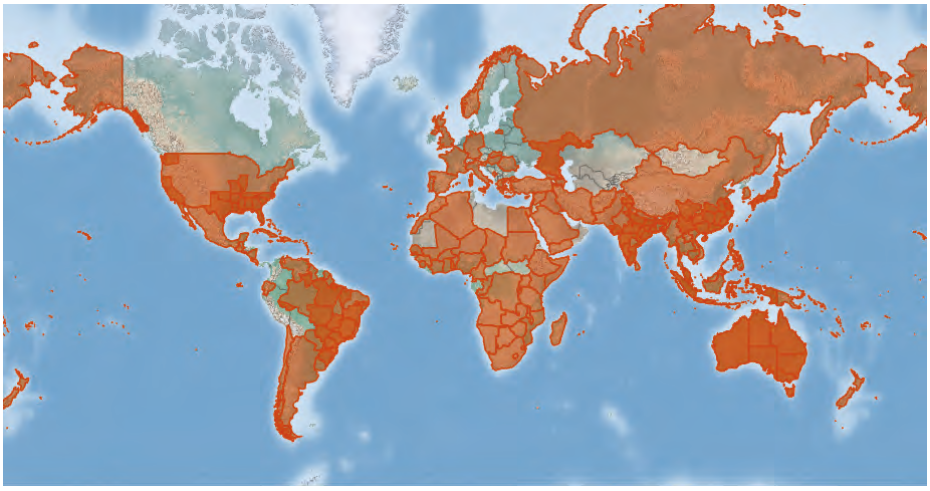

Figure 1.3. Current global distribution of *Nezara viridula* based on the Centre for Agriculture and Bioscience International (CABI) summary data. Where multiple records for a geographical area exist, CABI selects the most reliable status for the species in that area and marks it orange (CABI, 2024).

Dissertation outline

This PhD thesis delves into the tri-trophic interactions between *Nezara viridula*, its host plants and microbes, with an emphasis on the detoxification of plant secondary metabolites by symbiotic bacteria in the gut and salivary glands. Using a multi-omics approach to explore these interactions, this research seeks to advance our understanding of the relationships shaping insect-plant-microbe dynamics, offering valuable insights for pest management and ecological sustainability. Our main research questions were:

1. What is the composition of *N. viridula* microbiota?
2. Are symbionts essential for *N. viridula* survival and overcoming plant defences?
3. What are the factors that shape insect gut microbiota?

In **Chapter 2**, the current literature on microbial degradation of plant toxins is assembled in an overview article and knowledge gaps in this field are outlined.

Chapter 3 delved into the characterization of the *N. viridula* microbiota community composition with the determination of microbial role in overcoming plant defences.

In **Chapter 4**, we determined the impact of microbiota removal via egg surface sterilization on insect survival and fitness as well as the nature of the obligate *Sodalis nezarae* symbiont, addressing symbionts transmission route and presence of internal egg microbiome.

In **Chapter 5**, comparative genomics was used to analyze insect-associated *Commensalibacter* genomes including a novel *N. viridula* metagenome to determine microbial adaptations to symbiotic lifestyle.

Chapter 6 explored the involvement of *N. viridula* gut microbiota in detoxifying symbiosis and the influence of diet on gut microbiota composition to unveil factors that shape the insect gut microbial community.

Chapter 7 focused on the characterization of the *N. viridula* gut isolate *Pseudomonas* sp. Nvir which is capable of degrading the toxic secondary plant metabolite 3-nitropropionic acid. We cultured *Pseudomonas* sp. Nvir with

3-nitropropionic acid, to unravel the toxin breakdown pathway and determine whether it can be used as a growth substrate.

Lastly, **Chapter 8** provided a comprehensive integration of the thesis findings. It also discusses the potential implication of the knowledge gained in insect-plant-microbe interactions for pest management. The chapter includes a critical perspective of the conducted experiments and outlines directions for future research.

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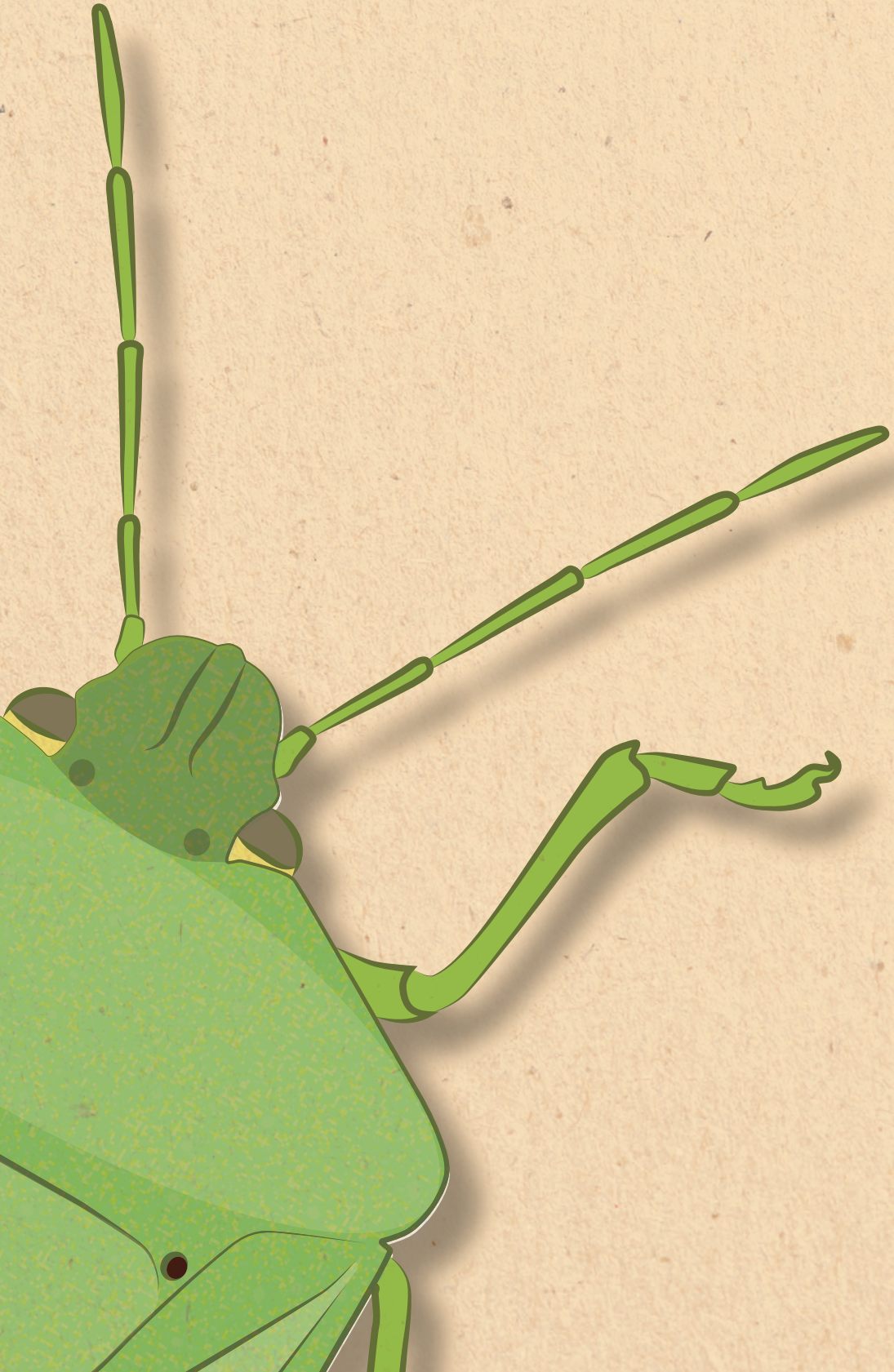

Chapter 2

Microbial degradation of plant toxins: a minireview

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Abstract

Plants produce a variety of secondary metabolites in response to biotic and abiotic stresses. Although they have many functions, a subclass of toxic secondary metabolites mainly serve plants as deterring agents against herbivores, insects or pathogens. Microorganisms present in divergent ecological niches, such as soil, water, or insect and rumen gut systems have been found capable of detoxifying these metabolites. As a result of detoxification, microbes gain growth nutrients and benefit their herbivory host *via* detoxifying symbiosis. Here, we review current knowledge on microbial degradation of toxic alkaloids, glucosinolates, terpenes, and polyphenols with an emphasis on the genes and enzymes involved in breakdown pathways. We highlight that the insect-associated microbes might find application in biotechnology and become targets for an alternative microbial pest control strategy.

Introduction

The *Plantae* kingdom includes organisms ranging from minuscule mosses to massive trees. Yet, regardless of their size, all plants produce a variety of low- and high-molecular-weight metabolites. According to their function, they have been classified into three categories: phytohormones (PHs), and phytochemicals, which include plant primary metabolites (PPMs), and specialized molecules or plant secondary metabolites (PSMs). PHs regulate metabolism and integrate internal and external signals to steer effective plant development and defence responses to counteract biotic and abiotic stresses (Aerts et al., 2021; Li et al., 2020; Pieterse et al., 2012). PPMs, namely carbohydrates, proteins, and lipids, are directly required for basic functions, such as photosynthesis, respiration, solute transport, nutrient assimilation, and biosynthesis of metabolic intermediates (Olivoto et al., 2017). Lastly, PSMs are metabolic products and intermediates, which are not essential for plant life or growth but instead, they navigate the interactions between plants and the surrounding environment (Davies, 2004; Erb & Kliebenstein, 2020; Heitefuss, 2010; Taiz et al., 2015). They are involved in inter-plant communication and the protection against herbivores, insects, and pathogens. They may also attract pollinators, seed dispersers, rood nodule bacteria, or influence oviposition, and in plant-plant and plant-microbe interactions act as communication signals (Hartmann, 1996; Wink, 2016). Thus, PSMs are essential in mediating plant adaptations to environmental changes.

PSMs are divided into several classes based on their chemical structure, including alkaloids, glucosinolates, terpenes, polyphenols, cyanogenic glucosides, amines, non-protein amino acids, polyacetylenes and fatty acids, polyketides, and carbohydrates (Wink, 2013). Approximately 200.000 PSMs have been found and around 100.000 have been experimentally investigated (Hartmann, 2007; Schoonhoven et al., 2005; Willis, 2017). The substantial number of different plant secondary metabolites are likely the results of the biochemical co-evolutionary arms-race proposed by Ehrlich and Raven in 1964 (Ehrlich & Raven, 1964). The theory suggests that plant-herbivore interactions in response to herbivore pressure drive plant evolution and diversification in biosynthesis pathways. Originally, PSMs derived from main precursor pathways *i.e.* acetate, shikimate, mevalonate, and deoxyxylulose, and diversification in these metabolic pathways lead to the generation of various PSMs (Ribera & Zuñiga, 2012).

As a result of constant environmental pressure, plants are genetically predisposed to continually synthesize diverse PSMs. Typically, plant genomes carry multiple gene families coding for enzymes that catalyse compound diversification from common precursors, allowing for a large diversification of PSMs. They benefit from modifying enzymes that can use multiple substrates and hence produce various products out of the same precursors. The structural diversity is even further increased by glycosylation and esterification, and occasionally by the co-modifications with PPMs (Dudareva et al., 2004; Kollner et al., 2004; Negre et al., 2003; Tholl et al., 2005).

Although plants synthesize a tremendous number of PSMs, the majority of PSMs are synthesized from primary metabolism and are accumulated in plant cells. The initial site of synthesis however is typically restricted to an organ, such as leaves, roots, or fruits, and subsequently, PSMs are transported and stored in destined plant tissues (Acamovic & Brooker, 2005). Storage preference differs per tissue or cell and in many plants the concentration of a particular compound varies between plant parts. In annual plants, they tend to concentrate in flowers, fruits, and seeds, whereas in perennial species they typically reach high levels in roots, bulbs, and stems (Guern et al., 1987). Some compounds were shown to be even stored in the epidermis (Wink & Roberts, 1998). The site of storage depends also on the compound's polarity, so hydrophilic compounds such as alkaloids, glucosinolates, and tannins are stored in vacuoles and idioblasts, whereas hydrophobic metabolites such as terpene-based compounds are stored in glandular hairs, trichomes, resin ducts, thylakoid membranes and on the leaf surface (Wiermann, 1981).

Plant secondary metabolites had significant contributions to human life. For centuries, they were used in various ways, especially in medicine as therapeutic painkilling and blood thinning agents (codeine, atropine), yet they also found application as dyes (indigo), flavouring additives (vanillin, mustard oils), fragrances (essential oils), stimulants (caffeine, nicotine), hallucinogens (morphine, cocaine), insecticides (anabasine piperine) and poisons (strychnine) (Heitefuss, 2010). Although PSMs can be toxic to humans and other animals, they are oftentimes eliminated from the environment *via* natural degradation by microorganisms. Since the emerging trend towards limiting the usage of pesticides in agriculture, PSMs, and their insecticide and herbicide properties have gained more interest. Using PSMs to restore sustainable crop protection could be thus an alternative solution to pesticides, which nowadays are less effective and contribute to pollution (Ahmad et al., 2022; Almeida et

al., 2017; Gangola et al., 2022; Itoh et al., 2014; Itoh et al., 2018; Schwarz et al., 2022; Singh & Singh, 2016; van den Bosch & Welte, 2017). Therefore, obtaining more insights into the microbial degradation of PSMs has great value to both agriculture and bioremediation.

In this minireview, we highlight the knowledge gaps in terms of natural plant-derived insecticides and herbicides and their application in resistance breeding (Figure 2.1). We will summarize the current research in the field of degradation of toxic PSMs, including alkaloids, glucosinolates, terpenes, and polyphenols, with a focus on microbial metabolic pathways and involved enzymes. We will provide evidence that toxin-degrading microorganisms are found in various ecosystems and, we will emphasize that instead of trying to eliminate microbes, we should profit from their degrading capabilities and apply them in biotechnology and bioremediation (Chen et al., 2019; Chitwood-Brown et al., 2021; Wille et al., 2019). We hypothesize, that resistance breeding focussed on plant secondary metabolites could greatly benefit sustainable pest control in the future. Ultimately, we will suggest in which direction research regarding increasing the effectiveness of biological pest control could continue to elucidate novel microbial pest control management strategies.

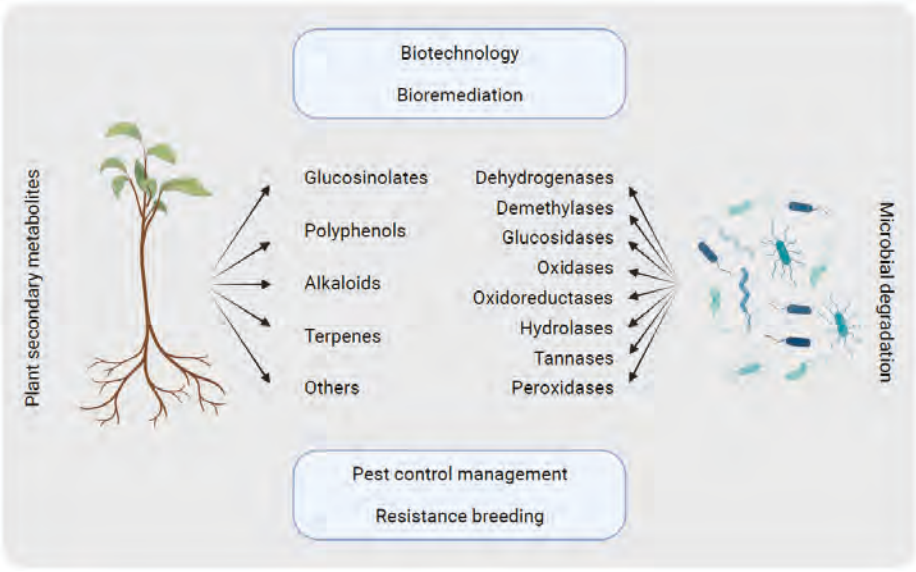

Figure 2.1. An overview showing the summary of microbial degradation of different secondary plant metabolite classes and their applications in agriculture, biotechnology, and bioremediation. Created with BioRender.com

Microbial degradation of toxic plant secondary metabolites

Plants produce various plant secondary metabolites (PSMs) in response to biotic and abiotic stresses. Here, we will focus on toxic PSMs and their degradation by environmental microorganisms. The number of toxic PSMs is substantial, which is why in this review, we will divide them into nitrogen-containing and non-nitrogen-containing PSMs.

Nitrogen-containing plant secondary metabolites

Alkaloids

Alkaloids are the most diverse heterocyclic nitrogen-containing PSMs. The occurrence of alkaloids was reported to be restricted to higher plants, but their production has also been confirmed in fungi (Builders, 2019). The chemical reactions catalysed by modifying enzymes, including methylation, glycosylation, oxidation, reduction, hydroxylation, and acylation led to the elucidation of approximately 27,700 different metabolites. The wide chemical diversity of alkaloids contributed to alterations of their physical, chemical, and biological properties and for this reason, there are three classification systems of alkaloids. The first one categorizes them according to their amino acid precursors *i.e.* phenylalanine, tyrosine, tryptophan, ornithine, lysine, histidine, and anthranilic acid (Wink, 2016). According to their chemical structure, alkaloids are grouped into heterocyclic and non-heterocyclic alkaloids, based on the position of the nitrogen atom in the chemical structure, and in the taxonomic division, alkaloids produced by plant species of the same genus are grouped under one category (Bhambhani et al., 2021). Selected toxic alkaloids are illustrated in Figure 2.2A-D.

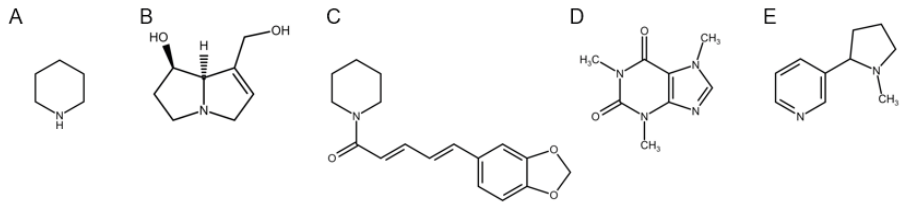

Figure 2.2. Chemical structures of toxic alkaloids and plant genus/species containing these alkaloids; **A)** piperidine (black pepper), **B)** retronecine (genus *Senecio*), **C)** piperine (black pepper), **D)** caffeine (coffee bean), **E)** nicotine (tobacco).

Alkaloids are oftentimes used in the pharmaceutical industry, however, their primary function in the plant is the activity against herbivores, insects, and pathogens and for that reason, anabasine is used as insecticide (Seigler & Seigler, 1998). Microbial transformation and degradation of alkaloids have been reported in various alkaloid classes.

Pyrrolizidine alkaloids (PAs) and PA *N*-oxides are examples of hepatoxic alkaloids identified in over 6000 plants, widely distributed in the Boraginaceae, Asteraceae, and Fabaceae families (Fu et al., 2004). At the moment, more than 660 PAs have been characterized with an estimation that 3% of the world's flowering plants contain PAs (Smith & Culvenor, 1981). Rumen microorganisms from naïve ruminants completely degraded monocrotaline within 48 hours *in vitro* (Aguiar & Wink, 2005), and a mixed culture of ovine ruminal microbes, including strain *Peptostreptococcus heliotrinreducens,* has been shown to degrade macrocyclic PAs from the plant common ragwort (*Senecio jacobaea*) to 1-methylene-containing compounds (Hovermale & Craig, 2002). The enzymes involved in the degradation pathway, however, remain unknown. Interestingly, it has been hypothesized that there might be a general biodegradation pathway of piperidine alkaloids. A mutant of *Mycobacterium smegmatis* mc2155 degraded for example non-alkaloid metabolites as well as piperidine and pyrrolidine alkaloids which shared similarities in their backbones (Poupin et al., 1999). The reaction was carried out by cytochrome P450 monooxygenase, which likely causes cleavage of the C-N bond and leads to the formation of intermediary amino acids. The reaction is followed by deamination and oxidation to a diacid and ultimately resulting in the complete mineralization of piperidine (Combourieu et al., 2000). In *Pseudomonas* sp. the piperidine alkaloid is first glutamylated and hydroxylated in the second transformation step, which is different than in *Mycobacterium* sp. *Pseudomonas* sp. KU43P converted, for example, piperidine into γ-glutamylpiperidine by a γ-glutamylpiperidine synthase, encoded by the *pip*A gene, which was further transformed by cytochrome P450 monooxygenase, γ-glutamyl-γ-aminovaleraldehyde dehydrogenase, γ-glutamyl peptidase, and γ-aminovalerate transaminase to glutaric acid. The corresponding genes are clustered together in part of the *pip* operon (Yamamoto et al., 2020).

Caffeine is a purine alkaloid and in plants, it serves as a toxic PSM against herbivores (Nathanson, 1984; Wright et al., 2013). Caffeine exhibits a negative effect on insects, arachnids, slugs, and snails and is therefore considered a natural pesticide (Abdelkader et al., 2013; Hollingsworth et al., 2002). Currently, two pathways for bacterial caffeine degradation are known: 1) *N*-demethylation

and 2) C-8 oxidation (Figure 2.3A). The enzymes of the two corresponding pathways share little similarity, which is reflected by substantially different intermediate products. Caffeine degradation *via N*-demethylation was observed by the microbiota of the coffee berry borer *Hypothenemus hampei* which infests coffee plants and shows no signs of intoxication. The pure isolate *Pseudomonas* sp. from the gut of *H. hampei* degraded caffeine *in vitro* with caffeine demethylase (NdmA; N_1-demethylase specific for N_1-methyl group of caffeine), encoded by *the ndm*A gene. This caffeine-degrading *Pseudomonas* sp. could use caffeine as a sole carbon and nitrogen source (Ceja-Navarro et al., 2015). Strain *Pseudomonas putida* CBB5 additionally harbours the *ndm*B gene which encodes the NdmB N_3-demethylase specific for the N_3-methyl group of theobromine, a first transformation product of caffeine. In the *N*-demethylation pathway, generally, NdmA demethylates caffeine to theobromine, and subsequently, NdmB demethylates it to 7-methylxanthine. Ultimately the pathway yields glyoxylic acid and urea (Summers et al., 2012). On the contrary, caffeine degradation *via* the C-8 oxidation pathway has been demonstrated in *Pseudomonas putida* and *Serratia marcescens* isolated from a coffee plantation soil, and coculture of *Klebsiella* sp. with *Rhodococcus* sp. In *Alcaligenes* sp. isolated from lake water, caffeine was degraded with a serine-type metallo-caffeine oxidase (Dash & Gummadi, 2010; Madyastha & Sridhar, 1998; Mohapatra et al., 2006). The study by Mohanty et al. (2012) revealed that *Pseudomonas* sp. strain CBB1 detoxified caffeine with a novel trimeric caffeine dehydrogenase (Cdh), encoded by the *cdh*ABC operon, and was capable of growth on caffeine as the sole carbon, nitrogen, and energy source. In C-8 oxidation, caffeine is oxidized at the C-8 position to form 1,3,7-trimethyluric acid (TMU), which is then transformed into glyoxylic acid, dimethylurea, and monomethylurea. Although two pathways have been described for caffeine degradation, little is known about the enzymes involved in caffeine transformation. More questions are to be addressed regarding the possibility of C-8 oxidation of *N*-demethylated metabolites and the distribution of caffeine-degrading genes among bacteria. Here, we performed a phylogenetic analysis of the genes encoding NdmA, NdmB, and Cdh which are involved in two distinct degradation pathways of caffeine (Figure 2.3B). The analysis revealed that NdmA and NdmB share a high degree of similarity and are clustered closely, whereas Cdh forms a separate branch, showing dissimilarity between enzymes involved in *N*-demethylation and C-8 oxidation pathways of caffeine. Likewise, the similarities of the *Pseudomonas* spp. enzymes NdmA, NdmB, and Cdh were found in several other bacterial species, suggesting potentially widespread occurrence of caffeine detoxification among bacteria.

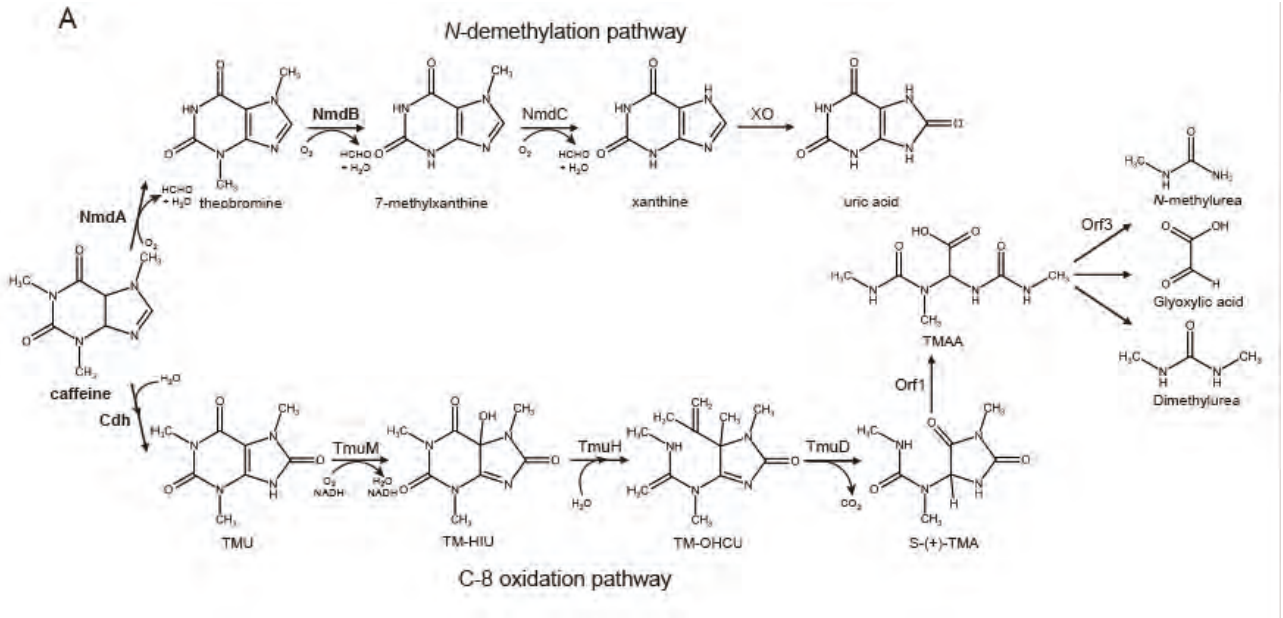

Figure 2.3A. Comparison of the *N*-demethylation and C-8 oxidation pathway of caffeine biodegradation and phylogenetic analysis of the key enzymes involved in the breakdown pathway. A) Two alternative degradation pathways of caffeine. (i) In the *N*-demethylation pathway caffeine degradation begins with demethylation at the N_1 position by methylxanthine N_1-demethylase (NdmA) forming theobromine. Subsequently, methylxanthine N_3-demethylase (NdmB) catalyses the removal of a methyl group at the N_3 position of theobromine, yielding 7-methylxanthine, which is further transformed to xanthine with N_7-specific *N*-demethylase (NmdC). Lastly, xanthine is metabolised to uric acid with xanthine oxidase (XO). (ii) In the C-8 oxidation pathway, caffeine dehydrogenase (Cdh) oxidises caffeine to 1,3,7-trimethyluric acid (TMU), which is then further oxidized to 1,3,7-trimethyl-5-hydroxyisourate (TMU-HUI) by trimethyluric acid monooxygenase (TmuM). TMU-HUI is metabolized to 3,6,8-trimethyl-2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (TM-OHCU) by 1,3,7-trimethyl-5-hydroxyisourate hydrolase (TmuH) and subsequently to 3,6,8-trimethylallontoin (S-(+)-TMA) *via* 3,6,8-trimethyl-2-oxo-4-hydroxy-4-carcoxy-5-ureidoimidazoline decarboxylase (TmuD) and 1,6,8-trimethylallantoic acid (TMAA) *via* a putative trimethylallantoinase (Orf1). Lastly, TMAA is cleaved to dimethylurea, glyoxylic acid, and monomethyl urea by acetylornithine deacetylase (Orf3). Degradation pathways are reconstructed based on the proposed caffeine-degrading pathways of *Pseudomonas putida* CBB5 and *Pseudomonas* sp. CBB1 (Mohanty et al., 2012; Summers et al., 2012).

Figure 2.3B. Comparison of the *N*-demethylation and C-8 oxidation pathway of caffeine biodegradation and phylogenetic analysis of the key enzymes involved in the breakdown pathway. B) Phylogenetic tree of *N*-demethylases and caffeine dehydrogenases. Multiple sequence alignment was constructed based on the protein sequence of the corresponding genes using Blastp search with default settings. The clustering of tree branches depicts differences in the proteins belonging to the *N*-demethylation and C-8 oxidation breakdown pathways of caffeine. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Nicotine is a toxic PSM produced by tobacco (*Nicotiana*) plants. Due to the processing of tobacco products, nicotine accumulates in soil, freshwater, and the plant rhizosphere, and contributes to the pollution of the environment (Jimenez et al., 2002). Although nicotine has toxic properties, nicotine-degrading microbes have been characterized. Oftentimes these microorganisms are applied in bioremediation to reduce nicotine pollution, *e.g. Arthrobacter* sp. follows a pyridine pathway, which attacks the nicotine pyridine ring during the degradation (Briški et al., 2003; Meher et al., 1995). *P. putida* S16, in contrast, degrades nicotine *via* an alternative pyrrolidine pathway. A key enzyme in this pathway is nicotine oxidoreductase NicA2 which converts nicotine to *N*-methylmyosmine. In subsequent degradation steps, the intermediate products are transformed ultimately yielding fumaric acid as the end product. In fungi, however, a demethylation pathway is used instead, which demethylates the pyrrolidine ring of the nicotine. Interestingly, in *Agrobacterium tumefaciens* strain S33 a novel pathway of pyridine and pyrrolidine degradation was found. *A. tumefaciens* transform nicotine *via* the pyridine pathway to 6-hydroxy-pseudomooxynicotine, through 6-hydroxy-L-nicotine and 6-hydroxy-N-methylmyosmide, and then *via* the pyrrolidine pathway to 6-hydroxy-3-succinoylpyridine and 2,5-dihydroxypyridine (Wang et al., 2012). Interestingly, the nicotine-degrading enzymes were encoded both in the genome and in plasmids which hinders the possible horizontal gene transfer of genes required for nicotine biodegradation to evolutionary distinct non-degrading microbes (Gurusamy & Natarajan, 2013; Tang et al., 2013; Uchida et al., 1983; Wang et al., 2009; Wang et al., 2012).

In potatoes (*Solanum tuberosum*) all parts of the plant produce two steroidal glycoalkaloids, α-chaconine and α-solanine (Figure 2.4A-B) which are toxic to humans, snails, insects, and fungi (Fewell & Roddick, 1993; McKee, 1959; Morris, 1984; Smith et al., 2001). Similar to nicotine, these toxic glycoalkaloids may leach into the groundwater after the decomposition of dead plants, causing a danger of acute poisoning in aquatic organisms. Jensen, Jacobsen, et al. (2009) found that groundwater microorganisms were capable of degrading glycoalkaloids α-chaconine and α-solanine to the corresponding β- and γ-structures and ultimately solanidine *via* stepwise removal of monosaccharides from the side chain. Similar intermediary product formation was found in two fungal potato pathogens from the genus *Gibberella* (Weltring et al., 1997). *Arthrobacter* sp. S41 isolated from potato field soil was able to degrade α-chaconine and α-solanine similarly *via* enzymatic activity of β-galactosidase, β-glucosidase, and α-rhamnosidase. This study showed that these genes form

a gene cluster encoded in the genome that harboured novel enzymes for the deglycosylation of potato glycoalkaloids (Hennessy et al., 2020). Overall, it seems that α-chaconine and α-solanine in both fungi and bacteria are degraded in the same 3-step breakdown pathway, generating β- and γ-structures of toxic glycoalkaloids, yielding in the final step solanidine. Whether a complete degradation of these toxic glycoalkaloids, namely further metabolism of solanidine is possible, remains however unclear.

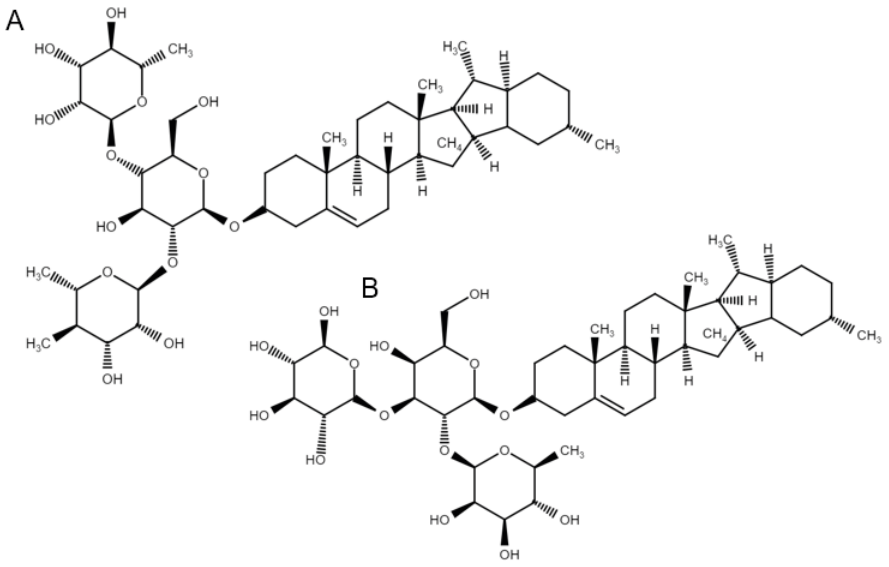

Figure 2.4. Chemical structures of toxic potato alkaloids; **A)** α-chaconine **B)** α-solanine.

Glucosinolates

Glucosinolates (GSLs) are nitrogen-containing β-thioglucoside-*N*-hydroxysulfates with a side chain and a sulfur-linked β-D-glucopyranose moiety. They are PSMs of the Brassicaceae, Capparaceae, and Caricaceae families, and currently, account for approximately 130 known PSMs (Fahey et al., 2001). Glucosinolates are present mainly in cruciferous vegetable crops, such as broccoli, cabbage, cauliflower, and turnip, and in non-cruciferous crops such as rapeseed (Bischoff, 2016; Wink, 2016). GSLs *per se* are not toxic and they do not exhibit biological activity, however, upon hydrolysis carried out by the myrosinase enzyme (β-thioglucosidase), they are converted to pungent and toxic isothiocyanates (ITC), thiocyanates and nitriles.

This phenomenon is commonly referred to as the *mustard oil bomb* (Lüthy & Matile, 1984; Wittstock et al., 2004). Normally, GSLs and myrosinase are physically separated in plants but upon tissue damage, myrosinase comes in contact with GSLs, causing their rapid hydrolysis (Koroleva et al., 2000). The produced glucosinolates hydrolysis products (GHPs) possess bactericidal, fungicidal, nematocidal, and allelopathic properties, making them natural pesticides. Several bacterial species found in various ecosystems were capable of GHPs degradation, including ITC, one of the most bioactive and toxic GHPs.

Pest insects feeding on Brassicaceae family plants encounter toxic ITC but show no adverse effect, implying their ability to either resist or degrade ITC to non-toxic products. Recently, it became apparent that insects benefit from acquiring microorganisms that mediate toxic degradation, enabling insects to infest various crops (Sato et al., 2021). One detoxifying symbiosis was shown in the cabbage root fly larvae *Delia radicum*, which is a notorious pest feeding on roots and stems of rapeseed and cabbage. It was demonstrated that *Serratia* sp., *Pectobacterium* sp., *Acinetobacter* sp., *Providencia* sp., and *Pectobacterium* sp. were able to break down 2-phenylethyl isothiocyanate (2-PI; Figure 2.5) *in vitro*. Likewise, strains carrying the Drgb3 plasmids encoded SaxA, an isothiocyanate hydrolase, that catalyses the conversion of 2-PI (van den Bosch et al., 2020; van den Bosch et al., 2018; Welte, de Graaf, et al., 2016). Phylogenetic analysis showed that plasmid-encoded *sax*A genes were present in diverse bacterial species, showing that detoxifying genes are frequently transmitted between bacteria (Itoh et al., 2018). Another pest of rapeseed, the cabbage stem flea beetle *Psylliodes chrysocephala*, harboured *Pantoea* sp. in the gut which rapidly degraded ITC *in vitro* (Shukla & Beran, 2020). The antibiotic treatment resulted in a decreased abundance of microbes and loss of capability to detoxify ITC. The authors demonstrated that *in vivo*, insects could restore ITC degradation when the microbiota was re-established after treating beetles with antibiotics. The results indicate the wide distribution of ITC degrading capabilities in insect-associated microorganisms and the significance of the bacterial symbionts in the detoxification of toxic PSMs.

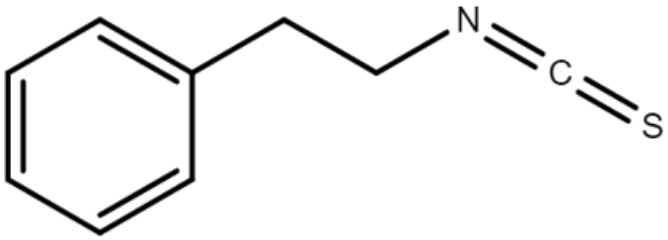

Figure 2.5.Chemical structure of 2-phenylethyl isothiocyanate which is widely present in cruciferous crops, such as broccoli, cabbage, and turnip.

Toxic ITC usually confers broad resistance against pathogens and herbivorous insects. Nevertheless, a fungal pathogen, the necrotrophic white mold *Sclerotinia sclerotiorum* was able to infect glucosinolate-producing-plants and eventually degrade ITC *via* either conjugation to glutathione or hydrolysis to amines (Chen et al., 2020). The importance of ITC-degrading microbes was also demonstrated in forest and nursery soils, where microbial degradation accounted for >60% reduction in the concentration of methyl-ITC (Zhang et al., 2005).

Other nitrogen-containing plant secondary metabolites

Alkaloids and glucosinolates are the two biggest classes of nitrogen-containing plant secondary metabolites, however, some nitrogen-containing metabolites do not belong to either class and therefore form a separate group. In this section, other nitrogen-containing PSMs are described.

Amygdalin (Figure 2.6A) is a cyanogenic glycoside that is typically found in honey bee-pollinated almond trees and microbe-mediated detoxification of amygdalin has been demonstrated in bees (*Apis* sp.). Among several amygdalin-degrading bacteria found in the bee's gut, *Bifidobacterium* wkB204 was capable of complete degradation to first prunasin and ultimately hydrogen cyanide *via* the activity of carbohydrate-degrading enzymes belonging to glycoside hydrolase family 3 (GH3). The amygdalin-degrading properties of GH3 were later confirmed by the expression of GH3 in *E. coli*, which resulted in the degradation of amygdalin to prunasin (Motta et al., 2022).

Another nitrogen-containing secondary plant metabolite, glycoside 3-nitropropionic acid (NPA; Figure 2.6B), is a toxic PSM produced by leguminous plants. NPA irreversibly inhibits succinate dehydrogenase in the TCA cycle, causing toxicity in eukaryotes. Microbial detoxification of NPA has been shown by soil microbes and gut-associated bacteria isolated from rumen and insects, like the Southern green shield bug *Nezara viridula* (Anderson et al., 1993; Nishino et al., 2010; Rogowska-van der Molen et al., 2022). Detoxification of NPA is carried out by either nitronate monooxygenase (NMO), encoded by *nmo*A or *pnm*R (putative nitronate monooxygenase [reductase]), or by propionate-3-nitronate monooxygenase (PnoA), encoded by *pno*A. Although three enzymes were identified to metabolize NPA, all transform it to a non-toxic intermediate 3-oxopropanoate with subsequent release of nitrate and nitrite. The reaction ultimately yields carbon dioxide and acetyl-CoA which enters the TCA cycle and thus serves bacteria as a carbon source (Rogowska-van der Molen et al., 2022). The phylogenetic analysis of genome-encoded *pnm*R revealed the widespread distribution of the gene in diverse bacterial classes, suggesting that potentially many NPA-degrading microorganisms remain yet uncharacterized (Rogowska-van der Molen et al., 2022).

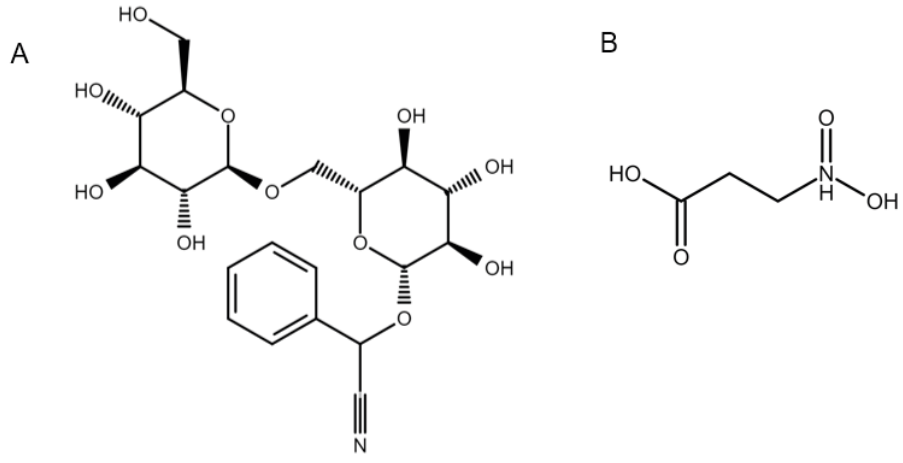

Figure 2.6. Chemical structures of A) amygdalin present in the almond tree and **B)** 3-nitropropionic acid (NPA) found in crown vetch.

Non-nitrogen-containing plant secondary metabolites

Terpenes

With more than 80.000 known compounds (Christianson, 2017), terpenes constitute the most chemically, structurally, and functionally diverse family of non-nitrogen-containing PSMs described to date (Christianson, 2017; Connolly & Hill, 1991). While these compounds can be synthesized by most organisms, they are particularly abundant and diverse in plants, being an essential component of tree resin and essential oils (Pichersky & Raguso, 2018). Terpenes consist of hydrocarbon chains (or rings) built from linked isoprene units that can subsequently be decorated with functional groups leading to the biosynthesis of terpenoids. Terpenoids are classified according to the isoprene units that contain either two, three, or four isoprene units *i.e.* monoterpenes, sesquiterpenes, and diterpenes, respectively (Figure 2.7A-C), and act as both primary and secondary metabolites.

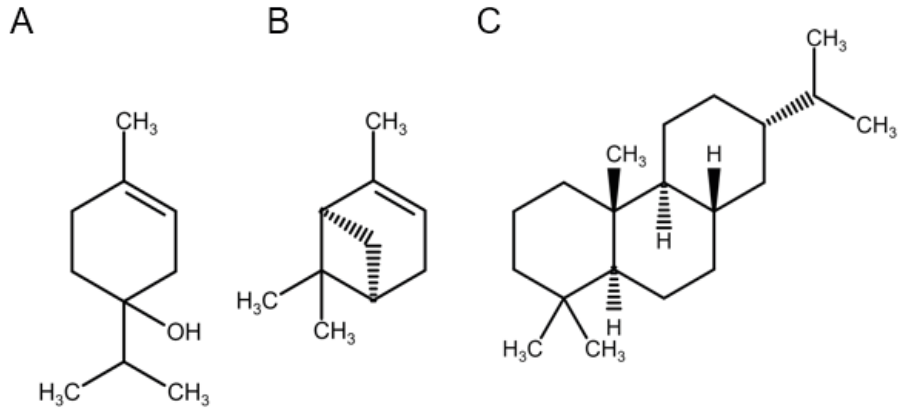

Figure 2.7. Chemical structures of terpenes present in pine tree; **A)** terpinene-4-ol, **B)** α-pinene, **C)** abietane.

For instance, pigments involved in photosynthesis or in maintaining membrane integrity such as carotenoids or sterols, are of terpenoid nature (Cazzonelli & Pogson, 2010; Dufourc, 2008). Most terpenes, however, serve plants as PSMs and increase fitness under abiotic or biotic stresses. Isoprenoids are known to mediate ecological interactions between plants and other organisms underlying attraction of pollinators, and most importantly, defence against pathogenic

microbes and herbivores, particularly insects (Bakkali et al., 2008; Gershenzon & Dudareva, 2007). Terpenoids thus contribute to both direct as well as indirect plant chemical defences. Direct defences frequently act as deterrents and can be toxic to herbivores through a variety of detrimental effects, hampering the normal functioning of herbivore metabolism. Indirect chemical defences, on the other hand, are used to attract herbivores' natural enemies such as predators or parasitoids (Delphia et al., 2007).

Although the exact mode of action of terpenoids remains unknown, some of their toxic properties derive from their lipophilic nature (Gershenzon & Dudareva, 2007). It has been determined, that by integrating between the acyl chains of phospholipids, terpenes damage cell membranes causing leakage of ions and metabolites (Keeling & Bohlmann, 2006; Lambert et al., 2001).

Given their protective role against natural enemies, many organisms have evolved mechanisms to cope with terpenes. Herbivores contend with isoprenoids in a variety of ways often involving behavioural strategies *e.g.* avoiding exposure by ingesting low amounts of these chemical defenses. For instance, bark beetle larvae feed gregariously in one continuous front, presumably outrunning tree-induced and terpene-rich resin (Deneubourg et al., 1990; Gregoire et al., 1981).

Microbes possess a variety of detoxification responses to terpenes (Marmulla & Harder, 2014). These strategies include i) excretion of terpenes through efflux pumps (Papadopoulos et al., 2008; Wang et al., 2013), ii) enzymatic detoxification of terpenes through glycosylation or oxidation (Wang et al., 2014), and, iii) utilization of terpenes as carbon sources for nutrition (Wang et al., 2014). The tea tree (*Melaleuca alternifolia*) has been used in traditional Australian medicine because it produces essential oils that are rich in monoterpenes with antimicrobial activity (*e.g.* terpinene-4-ol, 1,8-cineole, and α-terpineol). The bacterium *Pseudomonas aeruginosa* harbours an efflux system called MexAB-OprM that not only mediates its resistance against these compounds but is also responsible for its resistance to other antibiotics with clinical importance (Papadopoulos et al., 2008). On the other hand, *Pseudomonas putida* strain ATCC 17453 degrades the monoterpene camphor through a series of reactions, in which the first step is catalysed by a cytochrome P450 monooxygenase. The catabolic pathway results in the production of isobutanoyl-CoA and acetyl-CoA. The enzymes involved in these reactions are encoded by the operon *cam*ABCDEFG and are located in a plasmid. Lastly, free-living microbes isolated from pulp mill wastewater and forest soil, such as *Pseudomonas abietaniphila*

BKME-9 and *Burkholderia xenovorans* LB400, grow on a variety of diterpenes like abietane, a diterpene that commonly occurs on coniferous trees. These microbes harbour in their genomes a *dit* gene cluster, a group of 20 genes (as described in *P. abietaniphila* BKME-9) that are involved in diterpene catabolism. Not all genes within the cluster are required for diterpene mineralization, but at least three (*dit*I, *dit*H, and *dit*F) are essential (Martin & Mohn, 2000; Smith et al., 2004; Smith et al., 2007). Deleting *dit*R from the genome does not arrest the growth of *P. abietaniphila* BKME-9 on diterpene-rich media (Martin & Mohn, 2000), however, deleting *dit*Q impairs the growth of *P. abietaniphila* BKME-9 on dehydroabietic acid but not on abiotic acid (Smith et al., 2004). Not only can *P. abietaniphila* BKME-9 and *B. xenovorans* degrade diterpenes, they are also able to utilize them as their sole carbon source (Morgan & Wyndham, 2002; Smith et al., 2004; Smith et al., 2007).

Mutualistic terpene-degrading bacteria often live in a close relationship with insects (Itoh et al., 2018; van den Bosch & Welte, 2017). Herbivorous beetles feeding on coniferous trees profit from their gut microbiota, which detoxifies terpenes. Conifers are prolific producers of resin rich in mono- and diterpenes that are highly toxic to insects and have antibacterial properties (Bakkali et al., 2008). Nevertheless, many insects such as bark beetles and pine weevils are conifer specialists. Terpenoid catabolism by symbiotic microbes has been previously described for bacteria isolated from the gut of several bark beetles *in vitro* (Adams et al., 2013; Berasategui et al., 2017; Boone et al., 2013; Deneubourg et al., 1990; Xu et al., 2016).

The bark beetle *Dendroctonus valens* feeds on *Pinus tabuliformis* trees, whose resin is rich in α-pinene. Two yeasts and three bacterial strains isolated from the digestive system of this beetle can degrade pinene, lowering *in vitro* its concentration by half (Xu et al., 2016). Likewise, the mountain pine beetle *Dendroctonus ponderosae* hosts a bacterial gut microbiome dominated by *Pseudomonas* sp., *Rahnella* sp., *Serratia* sp., *Brevundimonas* sp., and *Burkholderia* sp. that harbour terpene-degrading genes (Adams et al., 2013). While *Serratia* sp. can reduce the concentration of all monoterpenes -except for α-pinene- by 80%, *Rahnella* sp. degrades up to 45% of the available α-pinene (Boone et al., 2013). Furthermore, both *Serratia* sp. and *Brevundimonas* sp. eliminate the diterpene abietic acid when it was present at low concentrations in the diet (Boone et al., 2013). While the mechanisms of monoterpene degradation in bark beetles remain undescribed, compelling evidence suggested that diterpene mineralization in the mountain pine beetle is catalysed by microbes

that harbour the *dit* gene cluster. Likewise, the bacterial gut metagenome in *D. ponderosae* is enriched in *dit* genes compared with that of other herbivores, suggesting these microbes may be benefiting the host through the detoxification of conifer defences (Adams et al., 2013). Further evidence of beetles benefiting from microbial degradation of terpenes comes from the large pine weevil (*Hylobius abietis*). The gut microbiome of this weevil is very similar to that of bark beetles, despite being phylogenetically more related to weevils such as the red palm weevil (*Rhynchophorus ferrugineus*) or the vine weevil (*Otiorhynchus salicicola*), specializing in palm trees and vines, respectively (Berasategui et al., 2016). A metagenomic survey of *H. abietis'* gut microbial community indicated that the microbiome of this insect harbours several *dit* genes (Berasategui et al., 2017). Genomic binning and subsequent phylogenetic analysis revealed that, as in other bark beetles, these genes are encoded in the genomes of members of Enterobacteriaceae strains (Berasategui et al., 2017). Consequently, the microbiome of the pine weevils can degrade diterpene both *in vivo* and *in vitro*. Thus, it is essential to further explore beetle-symbiont interactions, since it could provide insights in general understanding of role of detoxifying microbes in pest management.

Polyphenols

Polyphenols form one of the biggest and most complex classes of non-nitrogen-containing plant secondary metabolites of over 10,000 structurally different compounds that contain a hydroxyl functional group in the aromatic ring (Figure 2.8A-E) (Li et al., 2014). Polyphenols derive from the shikimate and malonic acid biosynthesis pathways, and are divided into four subgroups: phenols, phenolic acids, flavonoids, and tannins (Chiocchio et al., 2021; Olivoto et al., 2017; Teoh & Teoh, 2016). Polyphenols are present in all plant organs but individual groups have a storage preference. Phenolic acids, for example, are most often found in seeds, leaves, roots, and stems, flavonoids in aerial parts of plants, whereas tannins are often present in roots, bark, and seeds (Robbins, 2003; Tuominen et al., 2013). Polyphenols vary in size and structure, and can be either a single benzenic ring compound linked to a hydroxyl group (simple phenols) or benzoic acid derivatives (phenolic acids). More complex polyphenols, flavonoids, are composed of two benzene rings (A and B) linked by a three-carbon backbone (C_6-C_3-C_6; ring C), and an oxygen atom that forms a heterocyclic ring (L. Wang et al., 2022). Flavonoids are further classified into subgroups depending on the degree of saturation in the heterocyclic ring and can be either saturated (flavanones, dihydroflavonols, flavan-3-ols) or unsaturated (anthocyanidins, flavones, flavonols, isoflavones) (Cesco et al., 2012; Gahlawat et al., 2017;

Panche et al., 2016). Luteolin, tangeretin, quercetin, kaempferol, genistein and daidzein are one of the most known PSMs among flavonoids. Tannins, on the other hand, are high molecular weight PSMs, which are polymers constituted by flavonoid units or esterified monosaccharides with one or more molecules of phenolic acids (Bravo, 1998). They represent the fourth most abundant plant component, after cellulose, hemicellulose, and lignin (Lekha & Lonsane, 1997). Tannins are subdivided into two major groups based on their structures and properties: hydrolysable and condensed tannins. Condensed tannins are polymers composed of monomeric flavonoid units (flavan 3-ol or flavan 3,4-diol), which consist of two aromatic rings that are connected *via* C_4-C_8 bonds, as well as C_4-C_6 linkages in a three-carbon backbone chain but do not contain a carbohydrate core. Polymers of flavonol units are the most common type of tannins found in forage and browse legumes (Smith et al., 2005). Hydrolysable tannins, however, do not contain flavonoid units but are comprised of a polyol carbohydrate core, usually glucose, esterified to phenolic acids, such as gallic or ellagic acids, forming gallotannins and ellagitannins, respectively (Bule et al., 2020; Li et al., 2006).

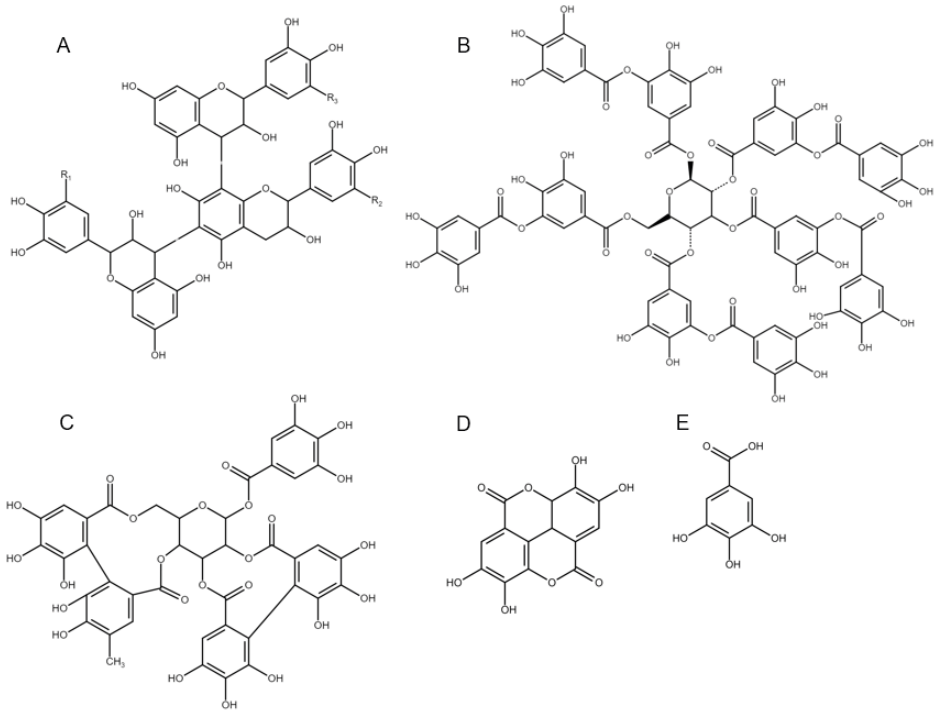

Figure 2.8. Chemical structures of selected polyphenols; **A)** condensed tannins, **B)** casurictin (ellagitannin), **C)** gallotannin, **D)** ellagic acid, **E)** gallic acid.

Polyphenols perform various functions in plants. Flavonoids, for instance, participate in floral pigmentation designed to attract pollinators. Flavonoids are additionally involved in UV filtration and can act as chemical messengers, physiological regulators, and cell cycle inhibitors. In host-microbe interaction, they can initialize the symbiotic relationship between Rhizobia and legumes (Galeotti et al., 2008; Kabera et al., 2014). Besides, polyphenols were proposed to serve as stabilizers of carbon in anoxic soils, according to the *enzyme latch* hypothesis (McGivern et al., 2021). Polyphenols that accumulate in anoxic soil are toxic to soil microorganisms which leads to the inactivation of their extracellular enzymes and ability to bind substrates, hence depriving microbes of nutrients and minimizing their microbial activity. This reduces the rate of soil organic matter decomposition (Fenner & Freeman, 2020; Freeman et al., 2001). Depending on the concentration of polyphenols in animal feed, they might be either beneficial or toxic. Low to moderate concentrations of tannins (≤4%) prevented bloating in ruminants, whereas high (>5%) concentrations inhibited ruminal gut microbiota and resulted in a reduction of nutrient digestibility (Smith et al., 2005). In addition, tannins showed a negative effect on insects (Goldstein & Swain, 1965), and high doses of quercetin (2-4%) led to chronic nephropathy in rats (Dunnick & Hailey, 1992). Hydrolysable tannins and condensed tannins were shown to have antinutritional and toxic properties when ingested by animals (Acamovic & Brooker, 2005). Furthermore, tannins are known to have antimicrobial properties and they are therefore resistant to microbial attack and degradation (Sallam et al., 2021). The structure of condensed tannins confers a higher resistance to attack than hydrolysable tannins and thus, condensed tannins are more toxic to microorganisms (Aguilera-Carbo et al., 2005). However, there are a significant number of bacteria and fungi that are resistant to these compounds and can degrade them to use them as a sole carbon source. Tannic acid is a high-molecular-weight polyphenol present in the seeds of *Camellia oleifera*. The camellia weevil *Curculio chinensis* is capable of overcoming plant chemical defences and degrading toxic tannic acid (Zhang et al., 2020). Zhang et al. (2020) showed that bacteria from the phyla Proteobacteria, Firmicutes, Fusobacteria, and Cyanobacteria were dominant degraders of tannic acid, however, the microbial enzymes involved in the degradation remain unknown. Likewise, the symbiotic yeast of the cigarette beetle *Lasioderma serricorne* was capable of tannic acid detoxification to gallic acid (GA) (Dowd, 1989). Furthermore, the rumen microbiota has been shown to harbour tannin-degrading microbes and the introduction of a tannin-adapted inoculum to Ethiopian Highland sheep prevented the adverse effect after feeding from *Acacia angustissima* which is rich in condensed tannins. The widespread

tannin resistance in rumen microbiota protects ruminant animals from antinutritional effects and is an example of a symbiotic relationship (Odenyo et al., 2001). Moreover, the introduction of tannin-degrading *Escherichia coli*, *Bacillus subtilis*, and *Enterococcus faecalis* bacteria from the desert woodrat to laboratory rats resulted in a higher body mass than control animals when exposed to tannins (Kohl et al., 2016).

The analysis of the degradation pathways of hydrolysable and condensed tannins showed that both subclasses do not share a common breakdown pathway. Hydrolysable tannins were found to be more easily hydrolysed than condensed tannins, due to the presence of ester bonds of gallic (gallotannins) or ellagic (ellagitannins) acids. Gallotannins and ellagitannins are considered the simplest forms of hydrolysable tannins, and upon hydrolysis, they yield gallic acid (GA), ellagic acid (EA), respectively, and glucose (Bhat et al., 1998). Ellagitannins are highly abundant in many plant species, and they occur in monomeric, dimeric, oligomeric, and C-glycosidic forms (Sallam et al., 2021). Although tannins are one of the most diverse classes of PSMs, hydrolysable tannins were found to be degraded by one ubiquitous enzyme, tannin acyl hydrolase, commonly known as tannase (EC 3.1.1.20). Tannase is present in various bacteria, fungi, and yeasts and catalyses the hydrolysis of ester and depside bonds (>2 monocyclic aromatic units linked by an ester group) in gallotannins, GA esters, epigallocatechin gallate, and epicatechin gallate, releasing GA and glucose (Aguilar & Gutiérrez-Sánchez, 2001; Bhat et al., 1998; de Las Rivas et al., 2019). Bacterial, yeast and fungal tannases share a common pentapeptide active site motif Gly-X-Ser-X-Gly, which is a common feature of the superfamily of esterases (Jimenez et al., 2014; Ren et al., 2013). Tannase is present in a diverse group of microorganisms that occupy different environments, such as rumen gut, soil, and wastewater. Bacteria that exhibited tannase activity are part of many genera e.g., *Actinobacillus* sp., *Campylobacter* sp., *Corynebacterium* sp., *Lactobacillus* sp., *Methanobrevibacter* sp., *Staphylococcus* sp., *Streptococcus* sp., *Streptomyces* sp. (de Las Rivas et al., 2019). Hydrolysable tannins, gallotannins, and ellagitannins are metabolised by tannase either under oxic or anoxic conditions, and the GA which is released upon tannase hydrolysis is further transformed into various metabolites. Under oxic conditions, *Pseudomonas putida* KR2440 metabolises GA as a sole carbon source *via* a ring-cleavage reaction, followed by hydration and final cleavage to pyruvic and oxaloacetic acid (Nogales et al., 2011). The authors found that enzymes involved in the degradation of GA are part of the *gal* gene cluster. On the contrary, anaerobic degradation of GA in *Lactobacillus plantarum* WCFS1 was

carried out by the oxygen-sensitive gallate decarboxylase forming pyrogallol as the intermediary product (Jimenez et al., 2013; Jimenez et al., 2014).

Even though hydrolysable tannins are a large group of polyphenols, their degradation is similar in many microorganisms, since the initial step relies on tannases. Tannases exhibit substantial differences in their molecular structures and amino acid sequences which likely is the adaptation to the complex structures of tannins. Moreover, tannase action is independent of oxygen availability and the differences in the formation of end-products from tannin degradation are restricted to the activity of the subsequent enzymes. The structural differences between hydrolysable and condensed tannins mean that the breakdown pathway of condensed tannins is not initiated by tannases. Below we discuss the current knowledge on the microbial metabolism of flavonoids and condensed tannins, which are polymers of flavonoid units and therefore share similarities in their breakdown pathways.

The gut microbiota of various animal species is well-adapted to the toxicity of condensed tannins and thus is capable of their degradation. The unique structure of flavonoid units (Figure 2.9A-C), which consists of A, B, and C rings, provides the chemical stability of condensed tannins and flavonoids. Their degradation mediated by microorganisms includes carbon-carbon cleavage reactions involving C- and A-rings, dehydroxylation, and hydrogenation and results in the formation of different compounds under oxic and anoxic conditions. The aerobic breakdown of flavonoid units is carried out *via* two alternative pathways and yields either quercetin or catechin. Quercetin is one of the most abundant flavonoids, predominantly present in fruits and vegetables in the form of *O*-glucosides (Anand David et al., 2016). Quercetin is converted by *Bacillus subtilis* 168 to 2-protocatechuoyl-phloroglucinol carboxylic acid and carbon monoxide by novel Fe-containing quercetin 2,3-dioxygenase encoded by the *qdo*I gene (Bowater et al., 2004). It is the first described prokaryotic carbon monoxide-forming enzyme that can utilize flavonol. On the other hand, the catechin breakdown pathway relies on the cleavage of the heterocyclic ring of catechin, a flavan-3-ol to phloroglucinol carboxylic acid and protocatechuic acid (Leisinger, 1981; William et al., 1986). The anaerobic conversion of catechin on the other hand yields diarylpropanol as a first degradation product, whereas quercetin is broken down into phloroglucinol and phenylacetate derivatives. Ultimately the anaerobic digestion of catechin and quercetin yields acetate and butyrate and depending on the microbial activity, acetate might either enter the TCA cycle, or along with butyrate be used in methanogenesis *via*

syntrophic ruminal microbes to form methane and CO_2 (Bhat et al., 1998; Field & Lettinga, 1992). *Bradyrhizobium japonicum* (Alphaproteobacteria) uses catechin as a sole carbon source (Hopper & Mahadevan, 1997) and it was found that condensed tannins are depolymerized to monomers, epicatechin, and catechin (McGivern et al., 2021). Another way to degrade flavonoids is a dehydroxylation reaction which is based on the removal of the *p*-hydroxy group from the aromatic ring. Human gut microbiota was shown to dehydroxylate ellagic acid into urolithins *via* sequential removal of hydroxyl groups (Espín et al., 2013). Hydrogenation of daidzein is the third way of flavonoid degradation and although daidzein is not toxic, its conversion is the model for understanding hydrogenation mechanisms of flavonoid units. Human gut bacterium *Slackia isoflavoniconvertens* (Coriobacteriia) converts soybean isoflavones daidzein and genistein *via* dihydrodaidzein and dihydrogenistein, respectively in subsequent hydrogenation reactions to equol and 5-hydroxy-equol (Schroder et al., 2013).

Phenolic acids and phenolic acid esters (Figure 2.9D-F) are the simplest and the last group of polyphenols. They are found to be the most toxic compounds in the polyphenolic class. Coumaric acid, benzoic acid, 4-hydroxybenzoic acid (4-HBA), vanillic acid, and 4-hydroxybenzaldehyde are among the best-known simple toxic phenolics. 4-HBA is commonly used in manufacturing processes (*e.g.* processing petroleum) which resulted in the accumulation of the compound in the environment. It is harmful to humans and the accumulation of 4-HBA in the soil causes deficiency of nutrients and inhibits the growth of plants. Two strains isolated from marine sediments, *Acinetobacter johnsonii* FZ-5 and *Klebsiella oxytoca* FZ-8 were able to degrade 4-HBA under anoxic conditions (Lu et al., 2022). Fungi *Phomopsis liquidambari* isolated from *Bischofia polycarpa*, degraded 4-HBA using three enzymes: 4-HBA hydroxylase, 3,4-dihydroxybenzoic acid decarboxylase and catechol 1,2-dioxygenase to *cis,cis*-muconic acid TCA cycle (Chen et al., 2011). Furthermore, rumen gut microbiota and fungus *Aspergillus niger* degraded toxic ferulic acid, caffeic acid, and coumaric acid, and multiple bacterial strains, including *Bacillus* sp., *Brucella* sp., and *Enterobacter* sp., isolated from tobacco cropping soil degraded *in vitro* eleven phenolic compounds (Chang et al., 2022; Kim et al., 2021; Lubbers et al., 2021). Likewise, the degradation potential for toxic phenolic compounds has been determined in the gut microbiota of the diamondback moth *Plutella xylostella*. The metagenomic analysis of *Enterobacter asburiae* and *Enterobacter cloacae* showed that these two species might aerobically degrade catechol. The authors identified eight genes encoding for catechol 1,2-dioxygenase, muconate

cycloisomerase, muconolactone D-isomerase, 3-oxoadipate enol-lactonase, 3-oxoadipate CoA-transferase, acetyl-CoA acyltransferase, 3-oxoadipyl-CoA thiolase, and 3-oxoadipate enol-lactose/4-carboxymuconolactone decarboxylase (Xia et al., 2017). The *in vitro* experiment showed that gut bacteria of *P. xylostella* degraded phenol within 24 hours. This is in line with findings from Kohl et al. (2014), establishing that the gut microbes of the desert woodrat *Neotoma lepida* are responsible for the detoxification of phenolic-rich resin from the leaves of creosote bush *Larrea tridentata* and antibiotic removal of microbiota resulting in woodrat susceptibility to toxic compounds.

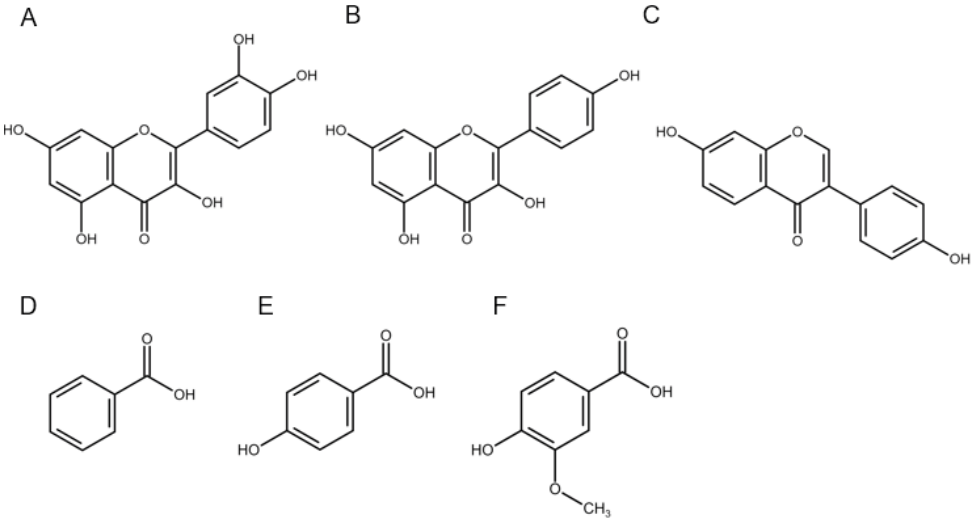

Figure 2.9. Chemical structures of flavonoids and phenolic acids and their occurrence in plants; **A)** quercetin (citrus fruits), **B)** kaempferol (kale), **C)** daidzein (soybeans), **D)** benzoic acid (cinnamon), **E)** 4-hydroxybenzoic acid (coconut), **F)** vanillic acid (*Angelica sinensis*).

Other non-nitrogen-containing plant secondary metabolites

Terpenes and polyphenols constitute the majority of non-nitrogen PSMs, and all other metabolites, that do not fit within either class and grouped separately. One such compound is oxalate, which is a hydrocarbon commonly present in higher plants. The calcium salt of oxalate (Figure 2.10) serves plants as a defensive agent against herbivores (Franceschi & Nakata, 2005). Genomic analysis of the

endosymbiotic bacterium *Ishikawaella capsulata* isolated from the plataspid shield bug *Megacopta punctatissima* showed that *I. capsulata* carries an *ode* gene in its plasmid the coding for an oxalate decarboxylase and therefore could have a detoxifying role in insects.

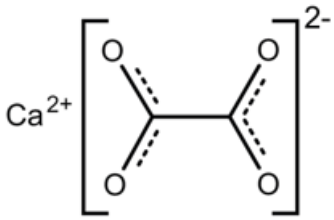

Figure 2.10. Chemical structure of calcium oxalate. It is accumulated in various plants, such as rhubarb or turmeric.

Degrading microbes in biotechnology and bioremediation

We have discussed the metabolic potential of microorganisms to degrade toxic PSMs. Microorganisms that exhibit toxin-degrading abilities may be applied in various industries, such as agriculture, biotechnology, and bioremediation. The identification of novel genes and determination of breakdown pathways allows understanding and optimization of the degradation processes. Moreover, these microbes might contribute to the removal of toxic PSMs from soil, aquatic sediments, and ground waters and similarly to the removal of pharmaceuticals, they might aid in the removal of plant toxins from wastewater.

Due to the increased usage of tobacco products, the industry generates solid and liquid tobacco wastes, which contain high concentrations of nicotine. The improper post-production handling of tobacco wastes causes nicotine to dissolve in water, which leads to the contamination of soil and groundwater. The microbial removal of nicotine from contaminated sources can be an effective way to decrease nicotine pollution in the environment (Gurusamy & Natarajan, 2013). A similar approach could be applied in handling tannery waste. Tannins are agricultural waste that exhibits antinutritional properties. They can bind proteins, making them unavailable for living organisms. Microorganisms were observed to grow and degrade tannins and therefore could be applied in waste management. Thus, tannin-degrading microbes could contribute to decreasing

tannin deposition in the ecosystem (Farias et al., 1994). Furthermore, several bacteria species were isolated from conifer pulp mill wastewater, which is rich in resin terpenes and their removal could prevent terpenes from leaching into the soil (Smith et al., 2008). On the other hand, moving towards sustainable waste management by recycling plant wastes using microbes seems to be crucial for a circular economy. Industrial plant wastes could be subjected to microbial valorisation since they are enriched in highly nutritious compounds. It was suggested that toxin-degrading microbes might contribute to the caffeine removal from coffee pulp and husk (Dash & Gummadi, 2006). These by-products are rich in carbohydrates and proteins after decontamination and could be further used as animal feed (Pandey et al., 2000). Moreover, the removal of α-chaconine and α-solanine from potato juice could result in the production of potentially high-value food ingredients due to the high protein concentration of potato juice (Hennessy et al., 2018). Also, *via* microbial decontamination, products containing nitrotoxins, such as NPA or 3-nitropropanol (NPOH) could be pre-treated with detoxifying bacteria or bacterial extracts (Rogowska-van der Molen et al., 2022).

Resistance breeding

As sessile organisms, plants developed well-adapted defences, such as the biosynthesis of PSMs that allow them to cope with environmental stressors like herbivores, insects, and pathogens. However, domesticated crops have lost many of their natural adaptive responses due to selective breeding directed towards favourable traits such as taste and appearance (Ku et al., 2020; Wink, 1988). To better protect commercial crops, plant breeders have started to reintroduce natural adaptive responses that are well embedded into the genetics of wild crop relatives and can be mined for plant breeding purposes (Huang & Han, 2014). Currently, natural genetic variation is explored using either wild plants, backcrosses, or inbred populations in large-scale genome-wide association studies and using quantitative trait loci mapping (Coolen et al., 2023; Coolen et al., 2019; Davila Olivas et al., 2017; Kloth et al., 2016; Proietti et al., 2018; Thoen et al., 2017). Ultimately these studies contribute to reintroducing lost plant defence mechanisms against pests.

Plant defences are regulated by plant hormones (PHs) such as salicylic acid, jasmonic acid, ethylene, abscisic acid, and a complex network of intertwined signalling cascades regulated by transcription factors (Frick et al., 2017;

Pieterse et al., 2012). Eventually, such defence cascades lead to the production of either constitutive or stress-induced PSMs. Some of these metabolites are healthy for humans, while others may be toxic to either humans or other animals, including pest insects, and pathogens. Examples of such secondary metabolites are quinolizidine-alkaloids that are abundant in lupins, making them thereby bitter and toxic to humans and insects, yet via cross-breeding sweet lupins were devoid of alkaloids, making them highly susceptible to insect herbivores (Wink, 1988; Wink et al., 1995). Since alkaloids are toxic, plant breeding is directed towards further reducing their content, thereby making plants more susceptible to insect herbivores.

Similarly, commercial tomato plants have lost their resistance to many insect herbivores, including virus-transmitting whiteflies. Wild tomato plants (*i.e.*, *Solanum habrochaites*) produce sesquiterpene 7-epizingiberene, which is toxic to spider mites, and repels whiteflies (Bleeker et al., 2011; Bleeker et al., 2012). Introgressing the sesquiterpene biosynthetic pathway of wild tomato into a cultivated tomato, resulted in improved plant resistance to several insect herbivores (Bleeker et al., 2011; Bleeker et al., 2012). Moreover, it also conferred resistance to several plant pathogens, including bacteria, fungi, and oomycete pathogens, showing the potential of PSMs in resistance to both insects and pathogens (Zabel et al., 2021). Incidentally, spontaneous mutations can lead to increased resistance against pathogens. In cotton, the flavonoid level was enriched and led to the red coloration of flowers and increased resistance to wilting caused by *Verticillium dahlia*, a major threat to cotton production (Long et al., 2019).

In grapevine, resistance to the powdery mildew pathogen *Erysiphe nectator* was associated with both plant primary and secondary metabolites (Ciubotaru et al., 2023). Metabolic profiles of susceptible and resistant plants pointed towards the involvement of many different compounds in plant resistance, including primary compounds, volatile organic compounds, and phenolic compounds. Complementary omics approaches will be necessary to reveal underlying genetics that can be used for resistance breeding. On the other hand, plants face the challenge of encountering multiple stress factors in the field that induce defense mechanisms that may counteract each other and therefore complicate breeding strategies (Coolen et al., 2022; Thoen et al., 2017). For these reasons, multi-stress and multi-omics approaches are of great value to further improve resistance breeding. Finding the ultimate combination of plant metabolites, with a focus on plant secondary metabolites, that confer resistance to major

threats while at the same time maintaining the crop's flavour and digestibility will hopefully support sustainable plant-based production in the future.

Microbial Pest Control strategies

The ability to overcome plant defences and degrade toxic PSMs is the evolutionary achievement of microorganisms. *Via* detoxifying symbiosis, microbes protect insects against the adverse effects of toxins. Insect-associated microbes show the ability to degrade toxic metabolites belonging to every class of secondary metabolites synthesized by plants. Their close relationship with the host contributes to the insects' widespread infestation of multiple plant species and poses a threat to the far-reaching spread of resistance to toxic compounds. The increase in the abundance of toxin-degrading microbes in the environment could lead to the overpopulation of pest insects, which could drastically reduce crop yields (Itoh et al., 2018; Rupawate et al., 2023). The Food and Agriculture Organization of the United States (FAO) estimated that approximately 40% of the world's total crops are lost due to the intervention of pest insects. Nowadays, the use of chemical pesticides has started to discontinue, and biological pest control strategies are time-consuming and oftentimes do not yield satisfactory results. Moreover, to secure the supply in the face of an increasing food demand an effective pest control strategy is needed. Manipulation of insect microbiota by targeting toxin-degrading microbes could become one of the approaches to fighting pests. Here, we emphasize that the Microbial Pest Control strategy (MPC) could become a sustainable and effective alternative to traditional pest management techniques.

Development of an alternative pest control strategy towards widely used chemical insecticides comes with challenges, however, microbes were already shown to be an effective tool in that field. One of the current MPC approaches relies on employing bacterial species as natural enemies against pests. The spores of an entomopathogen *Bacillus thuringiensis* (*Bt*) enhanced with bacterial culture broth suppressed the immune response of beet armyworm *Spodoptera exigua* (Hrithik et al., 2022). Further, *Photorhadbys luminescens* EGAP3 was found an effective biocontrol agent against the African migratory locust *Locusta migratoria migratorioides* (Muhammad et al., 2022).

Another MCP strategy specializes in targeting insect microbiota, which was shown to be essential for insects. Antibiotic removal of microbiota or egg

surface sterilization has negative effects on insects since they impair insects' development and fecundity (Goane et al., 2022; Kafil et al., 2013). Currently, one of the popular MPC strategies is the incompatible insect technique (IIT), which has been proven to be an effective approach in pest management and is used to manipulate insect microbiota, through which male insects are made incompatible for reproduction. These techniques are now widely used in *Drosophila* and mosquitos (Pagendam et al., 2020; Ridley et al., 2013). On the other hand, applying paratransgenesis, as the MPC technique, allows for the genetic modification of the insect gut microbes. The genetically modified microbes that are no longer capable of the synthesis of essential nutrients the insects cause a reduction in the insect population (Taracena et al., 2015). Targeting gut symbionts *via* CRISPR-Cas9 mechanisms is another novel MPC strategy, since it allows deleting of detoxifying genes and hence decrease the spread of resistance phenotype among insects (Sander & Joung, 2014; Selle & Barrangou, 2015; Zhao et al., 2020). Although several MPC approaches emerged, they often suffer from technical difficulties, such as population size in IIT, or high costs and limiting their use. More research needs to be done to tackle these obstacles and deliver sustainable and affordable means to fight pests and insects.

Over the past decades several alternative approaches focusing on exploiting microbes as MPC strategy have been described, yet only recently have they started to gain popularity. The manipulation of gut microbiota *via* IIT, paratransgenesis, CRISPR-Cas9, and their application in pest control are considered historic breakthroughs, and more research should be conducted in that direction. The development of novel MPS techniques could lead to securing future food demand and guaranteeing environmental safety.

Conclusions

Plants rely on the interdependent function of plant hormones, primary metabolites, and secondary metabolites. Although plant hormones and primary metabolites are essential for plants, their life depends on their capacity to interact with the environment and is largely facilitated by secondary metabolites. One of their main roles is providing defence against the harmful attackers such as herbivores, insects, and pathogens. This defence depends on the biosynthesis of toxic compounds, which have been classified into nitrogen-containing and non-nitrogen-containing PSMs. The most-studied PSMs belong to the classes

of alkaloids, glucosinolates, terpenes, and polyphenols, and even though they exhibit toxic properties, many microorganisms have the capabilities to detoxify or biotransform them into non-toxic compounds. Often, these toxins even serve microbes as nitrogen and/or carbon source. Toxin-degrading microbes were isolated from various ecological niches, such as soil, water, human, rumen, and insect gut systems, showing widespread detoxifying capabilities. The far-reaching distribution of toxin biodegradation by microbes poses a threat to ever-growing food demand and measures need to be taken to prevent further crop losses (Dwivedi et al., 2021). Studying these microorganisms might inform future crop protection approaches.

Microbes degrade compounds belonging to all major classes of PSMs, and often they rely on the same enzymes that detoxify different compounds. For instance, cytochrome P450 was shown to be involved in the degradation of both alkaloids and terpenes, and is commonly considered to be involved in the detoxification of various compounds in both eukaryotes and prokaryotes (Kelly & Kelly, 2013). On the other hand, some microbes harbour unique genes in their genomes and plasmids, and might even exhibit multiple detoxification pathways of the same metabolites. For example, bacteria belonging to the genera *Pseudomonas* sp. degrade toxic alkaloid caffeine *via* either *N*-demethylation or C-8 oxidation using breakdown pathways that showed little to no similarity, implying a divergent origin. Likewise, differences in the metabolic pathways are frequently dependent on the presence or absence of oxygen; the biotransformation of gallic acid yields different products under oxic and anoxic conditions. Even though the degradation of particular compounds may depend on many factors, the adaptive capabilities of microbes to degrade toxic plant secondary metabolites in the development of alternative microbial pest control and industry, have only recently gained more interest.

Many toxin-degrading microbes live in a close relationship with insects, and *via* detoxifying symbiosis, they provide them with protection against the adverse effect of toxic PSMs and insecticides (Itoh et al., 2018; van den Bosch & Welte, 2017). Henceforth, microbes contribute to the spread of insect resistance to natural plant defences. One of the trends in current pest control management is applying the breeding of domesticated plants with wild species to restore the lost potential of plants to defend themselves against insects and pathogens (Ku et al., 2020). Although challenging, focusing on the optimization of resistance breeding and choosing the right combination of metabolites might bring a functional solution to current problems in pest control. Alternatively, targeting

detoxifying microbes that live in a symbiosis with insects, is yet another example of benefiting from their evolutionary achievements. Recent scientific advances allowed for such techniques as incompatible insect techniques, paratransgenesis, and CRISPR-Cas9 to target specific microbes and therefore modulate the insect's microbiome (Rupawate et al., 2023). Ultimately, detoxifying microbes might be applied in biotechnology and bioremediation to pre-treat contaminated feed and remove toxic compounds from soil and wastewater (Jensen, Strobel, et al., 2009; Rogowska-van der Molen et al., 2022).

Acknowledgements

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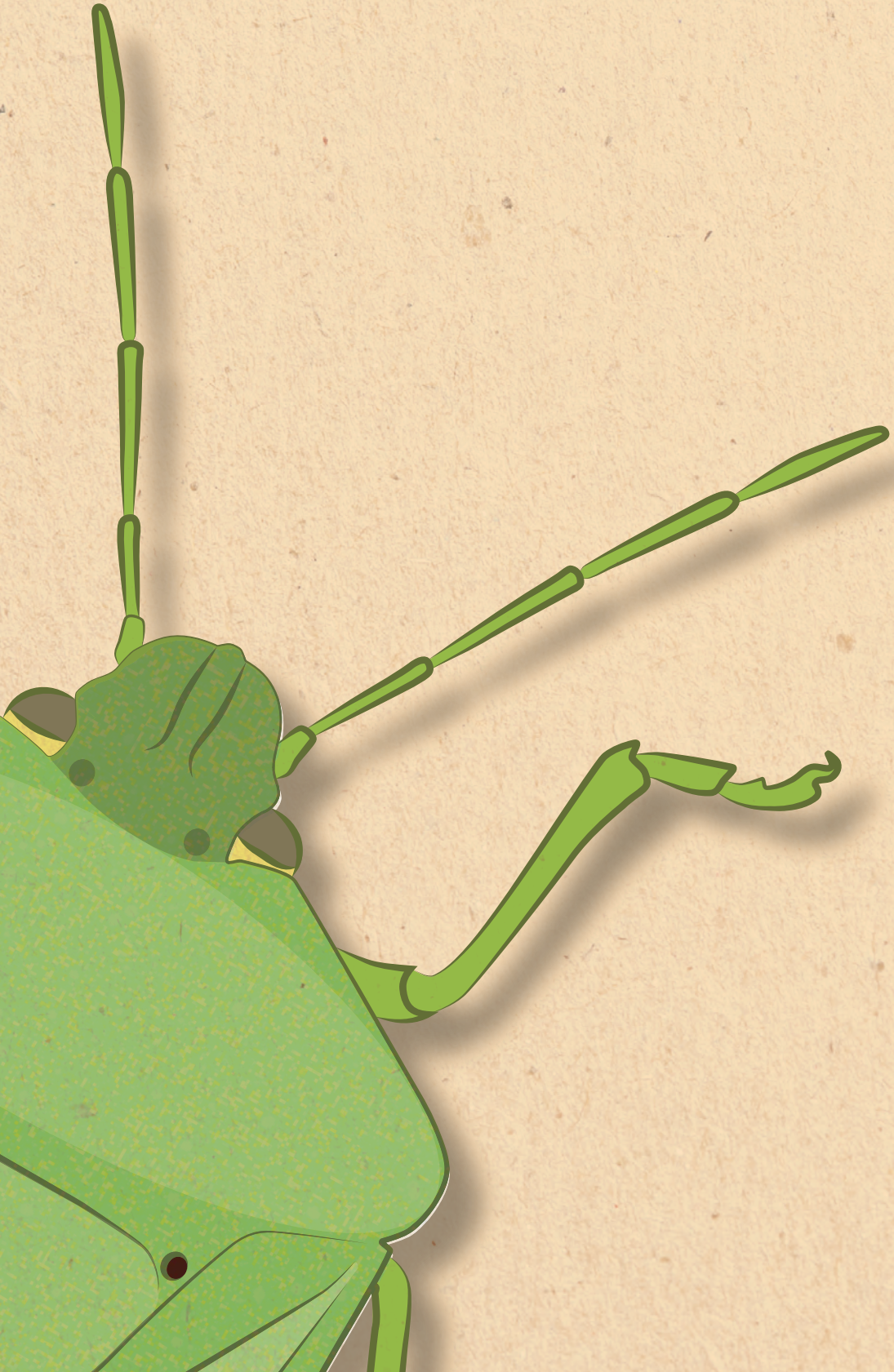

Chapter 3

Pest insect *Nezara viridula* microbiota mediates detoxification and plant defence repression

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Abstract

The Southern green shield bug, *Nezara viridula*, is an invasive piercing and sucking pest insect that feeds on crop plants and poses a threat to global food production. Since insects are known to live in a close relationship with microorganisms, our study provides novel insights into the community composition and function of the *N. viridula*-associated microbiota and its effect on host-plant interactions. We discovered that *N. viridula* hosts both vertically and horizontally transmitted microbiota throughout different developmental stages and their salivary glands harbour a thriving microbial community that is transmitted to the plant while feeding. The *N. viridula* microbiota was shown to aid its host with the detoxification of a plant metabolite, namely 3-nitropropionic acid, and repression of host plant defences. Our results demonstrate that the *N. viridula*-associated microbiota plays an important role in interactions between insects and plants and could therefore be considered a valuable target for the development of sustainable pest control strategies.

Introduction

Pest insects are among the major threats to global food production and cause significant crop losses (Harvey et al., 2003; Oliveira et al., 2014; Ribeiro et al., 1994; van den Bosch & Welte, 2017). Despite current pest management strategies, a third of the annually produced crops are lost due to pest insects and insect-transmitted plant diseases, and climate change is predicted to further increase these losses (Chakraborty & Newton, 2011; Deutsch et al., 2018; O'Hara et al., 2008; Sharma et al., 2017; Weintraub & Beanland, 2006). In addition, there is an increasing need for reducing pesticide usage to relieve environmental pollution and negative effects on non-target organisms. Together with a rapidly increasing human population, serious problems for food security are expected soon. Therefore, expanding our knowledge of how insects cope with plant defences is essential for the development of sustainable pest control strategies (Garrett et al., 2013; Newton et al., 2011).

To ward off insects, plants make use of structural barriers (*e.g.*, trichomes) and chemical defences (*e.g.,* toxic glycosides) that are constitutively present or induced upon recognition of potential threats. In the case of herbivorous insects, the induction of attacker-specific plant defences is triggered within seconds after wounding, leading to the production and accumulation of phytohormones and toxic secondary plant metabolites (Chauvin et al., 2013). Plant defensive phytohormones salicylic acid (SA) and jasmonic acid (JA) participate in defences against biotic threats, often acting antagonistically (Pieterse et al., 2012). Piercing and sucking insects, such as those from the order of Hemiptera, usually induce either one or both phytohormones resulting in the expression of downstream signaling genes such as pathogenesis-related 1 (*PR-1*) in the case of SA, and lipoxygenase 2 (*LOX2*) and myeloblastosis (*MYB*) transcription factors (e.g. *MYB28*) in case of JA (Giacometti et al., 2020; Proietti et al., 2018). Defence signalling eventually leads to the production of secondary metabolite chemical defences of which thousands are known, covering alkaloids, glucosinolates, polyphenols, and terpenes (Fahey et al., 2001; Rogowska-van der Molen et al., 2023).

Insects have coevolved with their host plants and harbour a wealth of adaptations that allow them to feed on plants with specialized defence mechanisms involving secondary plant metabolites (Jeckel et al., 2022; Li et al., 2009; Winde & Wittstock, 2011). Even though insects themselves adapted to coping with toxic compounds, they often acquire symbiotic microorganisms

that aid in breaking down toxic plant metabolites through detoxifying symbiosis (Almeida et al., 2017; Ceja-Navarro et al., 2015; Frago et al., 2012; Medina et al., 2018; van den Bosch & Welte, 2017; Welte, de Graaf, et al., 2016; Welte, Rosengarten, et al., 2016).

The Southern green shield bug, *Nezara viridula* (Pentatomidae: Hemiptera), is an invasive piercing and sucking pest insect that feeds on plant sap of plants, including crop species from the cruciferous, solanaceous, and fabaceous plant families that represent major food crops (*e.g.* tomato, beans (Todd, 1989)). As in other shield bugs, *N. viridula* has crypts in the M4 section of the midgut that carry species-specific symbionts that are vertically transmitted via egg surface contamination to the offspring (Prado et al., 2006; Tada et al., 2011). Geerinck et al. (2022) revealed that the egg-associated community of *N. viridula* consists of mainly Gammaproteobacteria and some Bacilli, specifically *Pantoea*-like symbiont, *Sodalis*, *Serratia*, *Niallia*, *Staphylococcus* and *Bacillus* strains and study on adult *N. viridula* showed the dominance of *Pantoea, Yokenella,* and *Enterococcus* in the midgut (Medina et al., 2018). *N. viridula* microbiota has been hypothesized to facilitate detoxification and repression of plant defences since insect-associated microbes transmitted while feeding caused vein necrosis in soybean seedlings (Medina et al., 2018; Ragsdale et al., 1979). Likewise, other insects have been described to transmit their microbiota and redirect plant defences to their benefit (Chung et al., 2013). In the case of the Colorado potato beetle (*Leptinotarsa decemlineata*), the transmission of microbiota during feeding represses plant JA-defences directed against chewing insects. This repression is caused by microbial SA induction that represses the plant's JA-defensive pathway via crosstalk (Caarls et al., 2015; Robert-Seilaniantz et al., 2011; Van Der Does et al., 2013).

To further explore the involvement of insect-associated microorganisms in insect-plant interactions, we studied the *N. viridula*-associated microbiota. The objective of this study is to characterize *N. viridula* core microbiota across different developmental stages and assess whether it supports plant defence repression and detoxification to the benefit of its host. To this end, we performed 16S rRNA gene and metagenome sequencing, microscopy of insect tissues, bacterial isolation, detoxification assays, and plant infection experiments, revealing that the *N. viridula* microbiota has a profound impact on plant-insect interactions.

Materials & Methods

Insect collection and rearing

N. viridula (42, 2nd and 4th instar nymphs) shield bugs were collected at Romeinenweerd in Venlo (Netherlands, 51.348028, 6.128802), on creeping thistle (*Cirsium arvense, Asteraceae*) on July 5th, 2019. Insects were transferred to greenhouse insect-rearing facilities with normal daylight and an additional 16-hour photoperiod with artificial light. Insects were allowed to feed on black mustard (*Brassica nigra*), black nightshade (*Solanum nigrum*), crown vetch (*Securigera varia*) and seeds of soybean (*Glycine max*), sunflower (*Helianthus annuus*), and brown mustard (*Brassica juncea*; Supporting Information Methods; Supporting Information Figure S1).

Insect dissection

To determine the core microbiota of *N. viridula* with 16S rRNA gene amplicon sequencing, eggs and whole 1st until 4th instars were used. From the 5th instar onwards, gut and salivary glands were dissected from insects and used either combined (for 5th instars) or separated for further analysis (n = 3; Supporting Information Figure S1, Table S1). Insects were dissected directly after submersion in 70% ethanol for one minute after which movement stopped. Dissection was performed under non-sterile conditions using a stereomicroscope, scalpel, scissor, and forceps. Separation of the complete gut system from the insect body was performed in phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) to prevent rupture of the tissue. Both salivary glands and complete gut systems were disrupted by vigorously pipetting or vortexing in lysis buffer for DNA isolation or PBS for culturing and isolation.

Saliva, frass, egg, and phyllosphere collection

N. viridula saliva was collected via a modified method described by Tan et al. (2016). Briefly, an artificial feeding solution was put onto a surface-sterilized watch glass, covered with parafilm allowing adult *N. viridula* to feed exclusively on it for 3 days. Afterwards, the solution was filtered and plated on Luria-Bertani (LB) agar (0.5% peptone, 0.3% yeast extract, 0.5% NaCl, and 1.5% agar; Supporting Information Methods). Insect frass was collected from adult *N. viridula* by pipetting deposited droplets directly into an Eppendorf tube (Supporting Information Figure S1). Sterilized egg clusters were obtained with 70% ethanol wash for 30 seconds and left to dry before DNA isolation. Phyllosphere was collected by 30 min PBS wash. For detailed instructions see the Supporting Information Methods.

Pure culture isolation and culturing of insect-associated microorganisms

Guts and salivary glands suspensions, saliva and frass samples were diluted (10, 100, 1000, and 10000 times) in PBS for CFU determination and plated on either LB or mannitol agar (2.5% *n*-mannitol, 0.5% yeast extract, 0.3% peptone, 1.5% agar) plates (Supporting Information Figure S2). Culturing was performed under aerobic conditions since anaerobic culturing lead to similar results. *Pantoea* was isolated from *N. viridula* gut systems on mannitol and LB agar after 2 days of incubation. *Serratia* was isolated from frass with an overnight culture on LB agar. *Sodalis* was isolated on LB agar from salivary glands samples and after 18 days growth at room temperature. Yeast was isolated from salivary glands by culturing on LB agar for 5 days. All isolated species were transferred to new LB agar plates at least six times before they were considered axenic. Bacterial density was determined with microscopy, using a Bürker-Türk counting chamber, and photometrically using a Cary 60 UV-Vis (Agilent) at 600 nm.

DNA isolation

Isolation of DNA for 16S rRNA gene and metagenome sequencing was performed using a DNeasy PowerSoil kit (QIAGEN, the Netherlands) including the optional heating step of the samples at 70 °C for 10 min in the Powerbead tubes. Tissue was directly put into the lysis buffer in the Powerbead tubes and tubes were vortexed for 10 min at 50 Hz using a TissueLyser LT (QIAGEN). DNA was eluted in 50 µL autoclaved ultrapure demineralized water and yield was measured with a Nanodrop1000 Spectrophotometer and a Qubit dsDNA HS assay kit (Thermo Fisher Scientific Inc., Waltham, USA). For detailed instructions see the Supporting Information Methods.

16S rRNA gene, metagenomic sequencing, and data analysis

16S rRNA gene amplicon sequencing (V3-V4 regions) was performed by Macrogen (Netherlands) with *Bac341F* and *Bac806R* primers (Caporaso et al., 2011; Herlemann et al., 2011) using an Illumina MiSeq sequencer (Illumina; Supporting Information Table S2). Paired-end (2x 301bp) reads libraries were prepared with the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina). 16S rRNA gene amplicon data was analyzed via our 16S rRNA amplicon sequencing pipeline.

Identification of pure cultures by 16S rRNA gene amplicon Sanger sequencing was performed by Baseclear (Netherlands) using the *Bac341F* and *Bac806R* primers and additional 18S rRNA *EUKA21F* and *EUKB1791R* primers (Medlin et al., 1988); Supporting Information Table S2). Raw Sanger sequencing

data was analyzed using the Chromas software with a subsequent sequence alignment of forward and reverse reads (excluding 18S rRNA reads that did not overlap) using EMBOSS Needle Alignment and an NCBI nucleotide megablast to determine the identity of the microbial isolates.

Template DNA extracted from guts and salivary glands was used for metagenome sequencing to obtain metagenome-assembled genomes (MAGs) of the *N. viridula* core microbiota. For detailed information regarding 16S rRNA amplicon sequencing, metagenomic sequencing, data and statistical analysis see the Supporting Information Methods.

Fluorescence *in situ* hybridization

Isolated gut and salivary glands were hybridized with a fluorescein-labeled general bacterial probe (Eub-mix (Amann et al., 1990; Daims et al., 1999)) and a Cy5-labeled Gammaproteobacterial probe (GAM42A (Manz et al., 1992) along with a GAM42A competitor probe. An additional Cy3-labeled probe specific for *Sodalis* sp. was used for targeted detection (Sod1238R (Koga et al., 2013). Samples were visualized using laser scanning microscopy. For a detailed protocol see Supporting Information Methods.

NPA degradation assay

Serratia was pre-grown aerobically in batch cultivation (n = 2) for 24 hours (30°C, 200 rpm) in M9 mineral salt medium (33.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.55 mM NaCl, 9.35 mM NH_4Cl, 4 mM glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, Thauer vitamin mixture (Sowers & Ferry, 1985), trace elements (Kurth et al., 2019), pH 7.2) until OD_{600} of 1.00 ± 0.05. Hereafter, 100 µM of NPA was supplemented to the pre-culture. For determination of NPA, nitrite, and nitrate concentrations, 1 mL samples were collected at 0, 2, 4, 6, and 24 hours (n = 2). The supernatant was collected by centrifugation at 20,000 × g for 3 min and was stored at -20°C until High-performance liquid chromatography (HPLC) analysis, and nitrite and nitrate determination (Supporting Information Methods).

Arabidopsis inoculation and insect feeding assay

One leaf per mature 5-week-old *Arabidopsis* plant (Supporting Information Methods) was inoculated with a 5 µL droplet of $1x10^8$ CFU/mL bacterial suspension in 10 mM $MgSO_4$, followed by a puncture with a sterile 0.4 mm needle through the inoculum into the leaf. Per treatment, 3-6 plants were inoculated or exposed to insect feeding. After 24 and 72 hours, plant leaves were harvested and directly put into liquid nitrogen after which they were stored at -70 °C for

RNA extraction. Each biological replicate consisted of 1-2 local (inoculated or feeding damage) leaves from different plants.

RNA extraction and cDNA synthesis

Plant RNA extractions were performed using a RNeasy Plant Minikit (Qiagen) according to the manufacturer, with the alteration that frozen plant material was ground with a micropestle directly in the extraction buffer. Approximately 150 ng/µL of RNA per sample was used for cDNA synthesis using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol with the prolonged incubation. RNA and cDNA qualities were determined with a Nanodrop.

N. viridula absolute microbial abundance

Real-time quantitative PCR (RT-qPCR) was performed to measure the absolute abundance of bacteria within samples. To quantify all bacteria, 16S rRNA gene primer pair *Bac341F* and *Bac806R*, amplifying V3-V4 regions was used. To determine the abundance of *Pantoea*, *Sodalis*, *Serratia*, and *Commensalibacter*, specific primer sets targeting single copy *gro*L and *rpo*B marker genes were designed (*PantF, PantR*. *SodF, SodR, SerF, SerR ComF, ComR*) based on the obtained MAGs form the metagenome analysis (Supporting Information Table S2). RT-qPCR was performed using a pipeline described in detail under the Supporting Information Methods.

Real-time quantitative PCR of plant cDNA

To determine the relative expression of both plant SA- and JA-defence pathways and the specific activity of the aliphatic glucosinolate secondary metabolite pathway, *A. thaliana* gene primers of *PR-1* (*At2g14610*), *LOX2* (*At3g45140*), and *MYB28* (*At5g61420*) were used (Supporting Information Table S2; (Giacometti et al., 2020; Proietti et al., 2018), comparing gene expression with the $2^{-\Delta\Delta CT}$ method to the housekeeping gene *PP2AA3* (Czechowski et al., 2005; Pfaffl, 2001). For detailed RT-qPCR methods see the Supporting Information Methods.

Results

Experimental approach

To characterize the core microbiota of *N. viridula* and subsequently unravel its role in insect-plant interactions we compared host plant leaf microbiota with insect gut systems, salivary glands, saliva, and frass samples using 16S rRNA gene amplicon sequencing (Figure 3.1). Fluorescence *in situ* hybridization was performed to visually localize *N. viridula* symbionts. Metagenome analysis was performed on adult insect gut systems and salivary glands to unravel the microbial metabolic potential. *N. viridula*-associated microbes were isolated and tested *in vitro* for degradation of plant toxins. Lastly, plant inoculations using isolated pure cultures were performed to determine the effect of *N. viridula* microbiota on host plant defences.

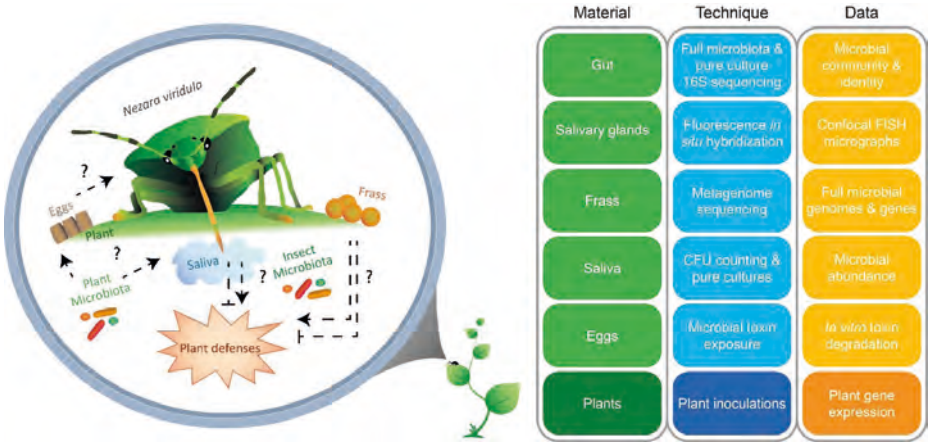

Figure 3.1. Experimental approach. An overview showing the experimental setup to identify *N. viridula* core microbiota throughout different developmental stages and subsequently unravel its role in insect-plant interactions. In the right panel the study materials (Supporting Information Figure S1), techniques used, and eventually obtained data are depicted.

N. viridula core microbiota consists of two main symbionts

The microbiota of *N. viridula* was determined by 16S rRNA gene profiling of all developmental stages. Single plant phyllosphere samples of black nightshade, black mustard, and crown vetch were taken along as a background for transient microbiota from ingested food. Of all *N. viridula* microbiota, the most abundant reads were assigned to the genus *Pantoea* followed by the genera *Sodalis*, *Serratia*, *Klebsiella*, *Commensalibacter*, *Pseudomonas* and *Cutibacterium* (Figure 3.2, Supporting Information Figure S3 and S4, and Tables S3 and S4).

Plant material contained minor numbers of bacterial reads belonging to the genera found highly abundant in *N. viridula*, indicating that transient microbiota ingested via plant material is not responsible for the high numbers of bacteria found in *N. viridula*.

Alpha diversity throughout the different developmental stages showed a steep and significant increase from egg to 3rd instar, indicating that young insects obtained diverse microorganisms from their environment. From the 4th instar onwards a significant alpha diversity decline is seen that can be explained by the fact that insect guts and salivary glands were dissected for these larger animals, whereas younger instars were used as a whole. From 4th and 5th instar to adult another significant decrease in alpha diversity is seen, that can partially be explained by the change in samples-type, in which the adult gut and salivary glands were separated (Supplemental Figure S3c, d, f and g).

Since 16S rRNA gene amplicon sequencing does not account for the variation in 16S rRNA gene copy numbers, absolute abundances of *Pantoea*, *Sodalis*, *Serratia,* and *Commensalibacter* were quantified in individual *N. viridula* adults, using RT-qPCR comparing 16S rRNA gene abundances with single-copy *gro*L and *rpo*B genes (Figure 3.3A). Similar to our amplicon sequencing results, *Pantoea* was the most abundant genus, followed by *Sodalis*, *Serratia,* and *Commensalibacter*. Variation in the absolute abundance within bacterial genera was minimal, indicating the stability of *N. viridula* microbiota. To confirm that aforementioned species are part of *N. viridula* core microbiota, 16S rRNA gene profiles obtained from adult insects collected from three other locations in the Netherlands were investigated and found to be in accordance with the microbial profile of our greenhouse-reared *N. viridula* (Figure 3.3B). The Bleiswijk sample shows the largest deviation from the other locations, which can be explained by extensive inbreeding of this particular research facility rearing population. Overall, all gut samples taken at different locations showed high abundance of *Pantoea* as found earlier.

Furthermore, we monitored the changes in the relative abundance of the most abundant bacterial genera throughout the different *N. viridula* developmental stages. Over 25% of the amplicon reads of *N. viridula* egg clusters belonged to the genus *Pantoea*, whereas *Sodalis* represented over 73% (Figure 3.2, Supporting Information Table S3). These numbers suggest that both *Pantoea and Sodalis* are vertically transmitted to *N. viridula* and therefore most likely fulfill essential symbiotic functions to their host. In addition, during the development

of *N. viridula* from egg to adult, a clear increase in the relative abundance of *Pantoea* was observed. Although *Sodalis* seemed to decrease during development, it could be found highly abundant in salivary glands, indicating that it serves a different role than *Pantoea*, which is predominantly found in the gut system (Figure 3.2,). All other genera showed a lower abundance throughout *N. viridula's* development. Our data demonstrate that *Serratia* is obtained by young 1st instar animals from the environment, and since its abundance in *N. viridula* is higher than that on plants, it seems *N. viridula* also supports the growth of this microorganism. Eventually, *Serratia* declined throughout the development of *N. viridula*, suggesting it is eliminated via an unknown mechanism or resides in organs that were not analyzed. *Klebsiella* was absent from the 1st instar and found in low abundance on the 2nd instar onwards, and is, therefore, most likely acquired from the environment. For *Commensalibacter* a similar lack of developmental pattern is observed with a small increase in abundance during the 3rd and 4th instar stage. *Pseudomonas* and *Cutibacterium* were detected in low abundance in both plants and *N. viridula*, suggesting that these genera are transferred via the environment or feeding. Our analysis also revealed that there is no clear difference in the microbiota profiles of adult males and females. Overall, our 16S rRNA gene sequencing results show that the *N. viridula* core microbiota is limited to two symbionts, namely *Pantoea* and *Sodalis*, and a limited consortium of *Serratia*, *Klebsiella,* and *Commensalibacter* with yet unclear relationships to their host.

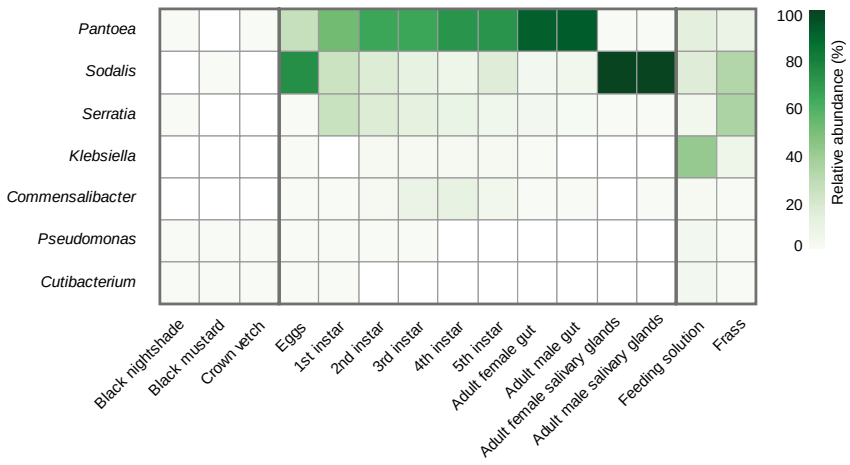

Figure 3.2. Microbiota of *N. viridula* over different developmental stages. Heatmap showing the relative abundance (in percentage) of the core genera (exceeding >10,000 reads in all insect-related samples, covering 89% (2,650,717) of all reads) within each sample type consisting of three biological replicates for *N. viridula* samples and one replicate for host plant samples (39 samples in total; Supporting Information Table S1).

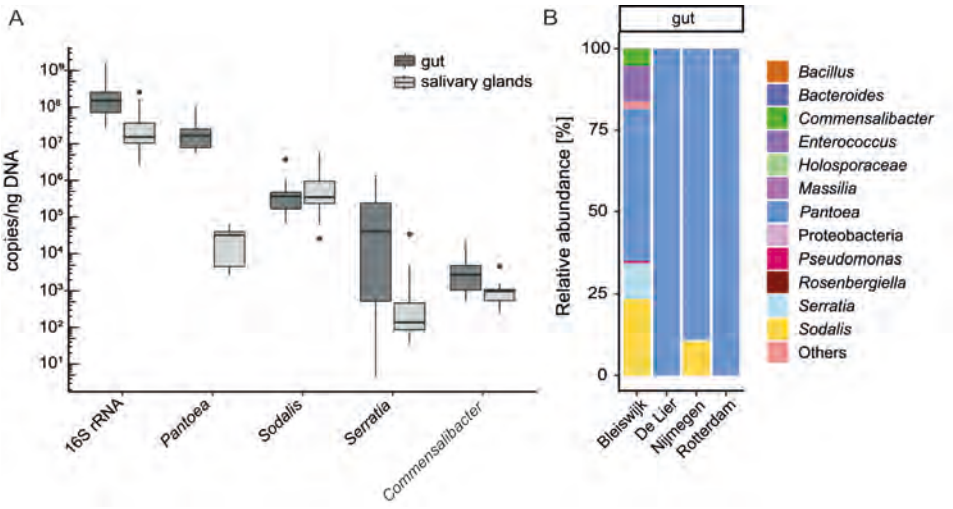

Figure 3.3. Absolute and relative abundance of *N. viridula* microbiota. **A)** A box plot showing the mean absolute number of 16S rRNA copies/ng (logarithmic scale) of 11 individual adult insects using RT-qPCR. 16S rRNA from either *Pantoea*, *Sodalis*, *Serratia,* or *Commensalibacter* was used to determine the number of copies compared to genera-specific single-copy genes *gro*L and *rpo*B. On the x-axis, the total number of 16S rRNA copies in the gut and salivary glands is shown with subsequent copy numbers for each genus. Error bars represent standard deviations and dots represent outliers. **B)** Core bacterial community composition of the gut based on 16S rRNA gene amplicon sequencing. The samples were compared between *N. viridula* adult insects obtained from four geographical locations in the Netherlands. Ten guts and ten pairs of salivary glands were dissected, pooled, and sequenced, and the taxonomy was displayed whenever possible at the genus level. Others represent the amplicon sequence variances (ASVs) that average below 1% of all reads.

N. viridula core microbiota is transmitted from insects to plants via frass and saliva

16S rRNA gene profiling of frass revealed that it contained approximately 10% *Pantoea*, 33% *Sodalis*, 36% *Serratia,* and 9% *Klebsiella* (Figure 3.2 and Supporting Information Table S3). Frass plated on LB agar resulted in approximately $1.8x10^8$ CFU/mL within a 24-hour incubation at room temperature (Supporting Information Figure S6). The genera *Sodalis* and *Serratia* were present in a relatively high abundance in frass, even though the gut system of the adult animals contained only low abundances of these bacteria. This could be explained by other organs that potentially harbour these bacteria such as Malpighian tubules, which are the extensions of the distal part of the gut system, hindgut (Coolen et al., 2022).

In contrast to earlier findings by Giacometti et al. (2020) indicated that *N. viridula* saliva obtained through ice exposure-induced salivation is sterile, our collected feeding solution offered to *N. viridula* contained excreted saliva with 13%

Pantoea, 16% *Sodalis*, 5% *Serratia* and 42% *Klebsiella* (Figure 3.2 and Supporting Information Table S3). Control feeding solution systems that were inaccessible to *N. viridula* remained sterile, suggesting that *N. viridula* transmits its associated microbiota during feeding. Culturing of collected feeding solution resulted in approximately $1.2x10^4$ CFU/mL of a particularly slow-growing microorganism on LB agar, which was later identified as yeast, highly similar to the genera *Wickerhamia* and *Candida* (Supporting Information Figure S6 and Table S5).

Taken together, 16S rRNA gene amplicon sequencing and culturing revealed that the *N. viridula* core microbiota is limited to several microorganisms of which some are transmitted via feeding, which might have consequences on insect-host plant interactions.

N. viridula salivary glands are colonized by Gammaproteobacteria and *Sodalis*

To visually confirm the localization of *N. viridula*-associated microbiota, we performed fluorescence *in situ* hybridization (FISH) on the gut system and salivary glands. We targeted all bacteria with fluorescent probes for Gammaproteobacteria and *Sodalis* and visualized them with confocal laser scanning microscopy. Microscopy images of the gut system confirmed that many rod-shaped Gammaproteobacteria were present in the cavities of the M4 midgut crypts of *N. viridula* (Figure 3.4A). Salivary glands of phytophagous shield bugs consist of a principal gland with anterior and posterior lobes, a hilum with a principal salivary duct, the duct of the accessory gland, and the accessory gland (Castellanos et al., 2017). Our analysis revealed that the entire principal gland tissue is colonized with Gammaproteobacteria and *Sodalis* is the dominant member of the community (Figure 3.4B and Supporting Information Figure S7).

Metagenome analysis reveals a detoxifying potential of *N. viridula* microbiota

The role and microbial metabolic potential of *N. viridula*-associated microorganisms was analyzed using metagenomics on *N. viridula* gut systems and salivary glands. Similar to the 16S rRNA amplicon data, the analysis revealed that *Pantoea* and *Sodalis* were the most abundant species in the gut and salivary glands, respectively (Figure 3.5 and Supporting Information S8). Moreover, we found that *N. viridula* gut and salivary glands harboured several strains of *Pantoea* and *Sodalis*. Further metagenomics analysis yielded four metagenome-assembled genomes (MAGs) of *Pantoea*, *Sodalis*, *Serratia,* and *Commensalibacter* (Table 3.1 and Supporting Information Table S1).

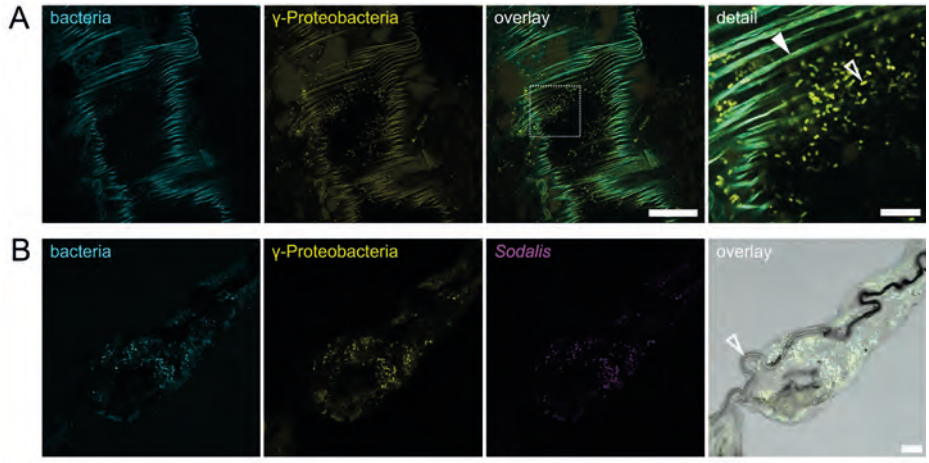

Figure 3.4. Colonization of *N. viridula* gut and salivary glands by Gammaproteobacteria and *Sodalis*. A) Confocal micrographs show the cavity of an adult *N. viridula* M4 gut crypt with FISH-stained microbes. Panel "bacteria" shows FISH-probe "Eub-mix" in cyan (Fluos dye). Panel "γ-Proteobacteria" shows FISH-probe "GAM42A" in yellow (Cy5 dye). Panel "overlay" shows the overlay of both channels. Scale bar "overlay" = 50 µm. A dashed square in "overlay" marks the location of the magnified panel "detail". Closed arrowhead points at autofluorescent gut structure. Open arrowhead points at FISH-stained bacterium. Scale bar "detail" = 10 µm. **B)** Confocal micrographs show an adult *N. viridula* salivary gland with the salivary duct. Panel "bacteria" shows FISH-probe "Eub-mix" in cyan (Fluos). Panel "γ-Proteobacteria" shows FISH-probe "GAM42A" in yellow (Cy5). Panel "*Sodalis*" shows FISH-probe "Sod1238R" in magenta (Cy3). Panel "overlay" shows the overlay of all three fluorescent channels merged with a transmitted-light brightfield channel. Open arrowhead points at salivary duct. Scale bar = 100 µm.

Table 3.1. Overview of the recovered MAGs. The information for each of the MAGs includes bacterial ID, genome size, GC content, completeness, contamination, number of contigs, and the relative abundance assigned based on the percentage of mapped reads. The relative abundance of unmapped reads was assigned to either mitochondrial or chloroplast reads and therefore was left out of the table.

Bacterial IDs (genus)	Genome size (bp)	GC content (%)	Completeness (%)
Commensalibacter	2009072	37.5	95.8
Pantoea	1426114	40.6	59.3
Serratia	5249834	59.8	99.8
Sodalis	4205851	56.7	100

[1]The abundance of the mapped reads within a sample.
[2]Sample isolated using a Blood&Tissue kit.

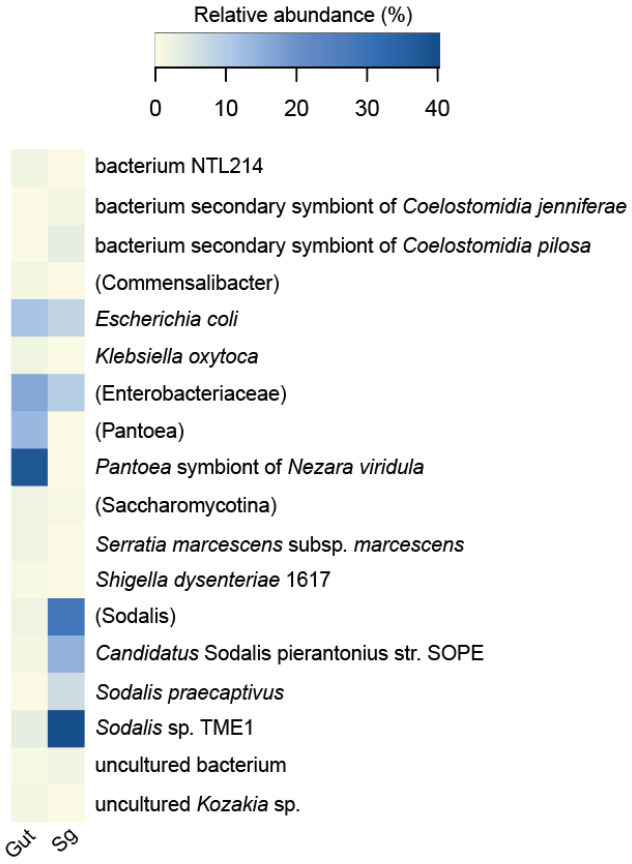

Figure 3.5. Relative metagenome gut and salivary gland microbial abundance. A heatmap showing the relative metagenome microbial abundance (> 1%) in adult *N. viridula* gut systems (Gut) and salivary glands (Sg), calculated percentage of reads based on raw reads from Blood&Tissue DNA isolation kit. When the species could not be assigned, the lowest taxonomic level was shown instead and was indicated with a taxonomic name between the parentheses.

Contamination (%)	Contigs (n)	Relative abundance (%)[1]	
		Gut[2]	Salivary glands[2]
0.0	145	2.5	0
0.0	12	57	0
0.5	91	9	0
0.0	189	7.3	68.5

N. viridula feeds on nutritious-poor and sugar-rich plant sap and relies on its symbiotic partners to biosynthesize deficient nutrients (Tada et al., 2011), therefore we analyzed the metabolic potential of *N. viridula*-associated microbes in terms of amino acid and vitamin metabolism and the degradation of carbohydrates (Supporting Information Tables S6, S7). *Pantoea*, *Sodalis*, and *Serratia* can biosynthesize most essential amino acids and all core microbes harbour partial vitamin B biosynthetic pathways, although none can biosynthesize thiamine or cobalamin. In terms of carbohydrate degrading properties, *Sodalis* harbours genes to degrade starch, and D-galacturonate (Supporting Information Table S7). A similar degradation potential was observed for *Serratia*, yet it lacked the genes to degrade D-galacturonate, a key constituent of pectin. *Commensalibacter* has the metabolic potential to degrade starch and, unlike others, might convert arabinan to L-arabinose and 1,4-beta-D-xylan to D-xylose, which are dominant in woody plants, plant seeds, and root components (Chandrasekaran, 1998; Córdova et al., 2019). *Pantoea* lacked most carbohydrate-degrading enzymes and therefore likely is not involved in the degradation of carbohydrates. In addition, both *Serratia* and *Sodalis* contained chitin degradation genes, which could support insect resistance to fungal pathogens (Ferrari & Vavre, 2011).

N. viridula microbiota could also provide its insect host with unique characteristics that may play a role in establishing symbiotic interactions with insects via cellular invasion, as well as the colonization of plants (Dale et al., 2002; Egan et al., 2014). We discovered that *Sodalis* encodes a complete bacterial type III secretion system (T3SS, *ysc*FCJRSTUVNQ), that is used by bacteria to secrete effector proteins, which can manipulate or even repress plant defences (Ceulemans et al., 2021). The latter can potentially reduce the production of anti-insect feeding compounds and toxic metabolites (Coolen et al., 2022; McHugh et al., 2019).

Since the insects used in this study were reared on native black nightshade and crown vetch plants that produce toxic α-solanine, α-chaconine, and 3-nitropropionic acid (NPA) metabolites, detoxifying symbiosis was investigated. α-Solanine and α-chaconine are two structurally related glycoalkaloids that inhibit cholinesterase activity and disrupt eukaryotic cells, and therefore are likely harmful to *N. viridula* (Jensen, Strobel, et al., 2009). *Serratia, Sodalis,* and *Commensalibacter* were confirmed to contain genes involved in α-solanine and α-chaconine degradation via β-galactosidase, β-glucosidase and α-rhamnose isomerase (Hennessy et al., 2020); Supporting Information Table S7). NPA is known to be toxic to eukaryotes by irreversibly inhibiting enzyme from the

tricarboxylic acid cycle (Rogowska-van der Molen et al., 2022). Detoxifying symbiosis by *N. viridula* symbiont *Serratia* by nitronate monooxygenase (*nmo*A), 3-oxopropanoate dehydrogenase (*bau*C), nitrite reductase (*nas*D) and flavohemoprotein (*hmp*) potentially supports its insect host with NPA degradation. Overall, genetic evidence points towards the possible involvement of *N. viridula* microbiota in the biosynthesis of nutrients, digestion of carbohydrates, suppressing plant defences and in detoxifying symbiosis.

Serratia degrades 3-nitropropionic acid

Serratia which harboured detoxifying genes was cultured with NPA for 24 hours to unveil whether *N. viridula* symbionts can degrade toxic plant metabolites. Rapid degradation of NPA was observed for the first 6 hours of incubation, after which the rate of degradation decreased (Figure 3.6). Simultaneously, nitrate and to a lesser extent nitrite by-product concentrations increased. No nitrate nor nitrite were present in control cultures, confirming that they are by-products of NPA degradation (data not shown). These results demonstrate the ability of gut-associated *Serratia* to degrade the toxic plant metabolite NPA.

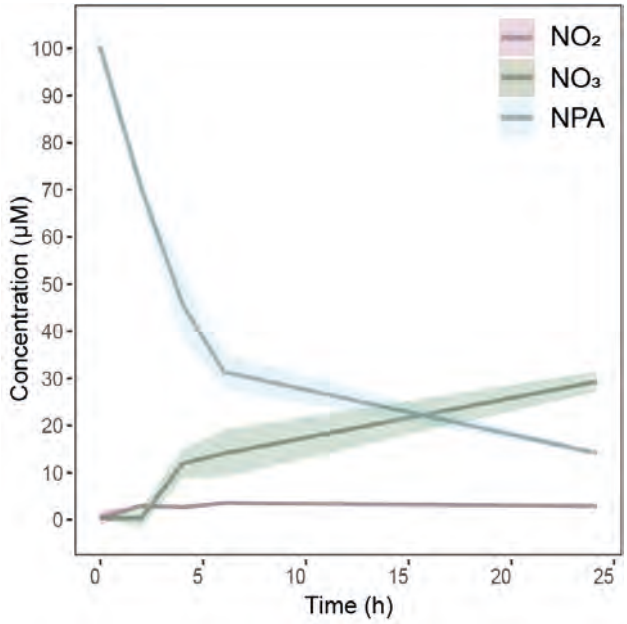

Figure 3.6. NPA degradation by *N. viridula*-associated *Serratia*. *Serratia* was grown in M9 medium until an OD_{600} of 1.00 ± 0.05. Hereafter, 100 µM of NPA was added, and samples were immediately taken (0 hours) and after 2, 4, 6, and 24 hours (n = 2). NPA (blue) was measured with HPLC and nitrite (NO_2^-, red) and nitrate (NO_3^-, green) concentrations were determined using a Griess assay. On the x-axis the time is shown in hours (h) and on the y-axis NPA concentration (µM). Shaded areas represent standard error.

N. viridula-associated microbiota repress plant defences

To determine whether *N. viridula*'s saliva and frass microbiota members, namely *Pantoea*, *Serratia*, *Sodalis*, yeast species could alter plant defences, they were inoculated on mature *Arabidopsis thaliana* plants (Brassicaceae). The results showed that when *N. viridula* fed on *Arabidopsis* for 72 hours, it significantly induced *PR-1* expression and suppressed *MYB28* expression (Figure 3.7 and Supporting Information Figure S9). We also observed that all tested *N. viridula* microbiota were able to significantly repress artificial piercing-induced *PR-1* expression at 24 hours after inoculation, raising the possibility that they also repress *N. viridula*-induced *PR-1* expression at 72 hours of feeding. At 72 hours of feeding, no *LOX2* induction was observed. On the contrary, at 72 hours, all tested *N. viridula* microbiota repressed artificial piercing-induced *LOX2* expression. JA-associated defence responses have previously been shown to be induced by *N. viridula* 3 hours after feeding by Giacometti et al. (2016). Taken together, the current data suggest that *N. viridula* microbiota repress both *N. viridula*-induced SA and JA pathways. *Sodalis* was the only microorganism that could significantly repress *MYB28* expression at 72 hours after inoculation, thereby potentially directly suppressing the plant's aliphatic glucosinolate defence pathway. In conclusion, our hypothesis that *N. viridula*-associated microbiota supports their host by counteracting insect-induced plant defences was confirmed.

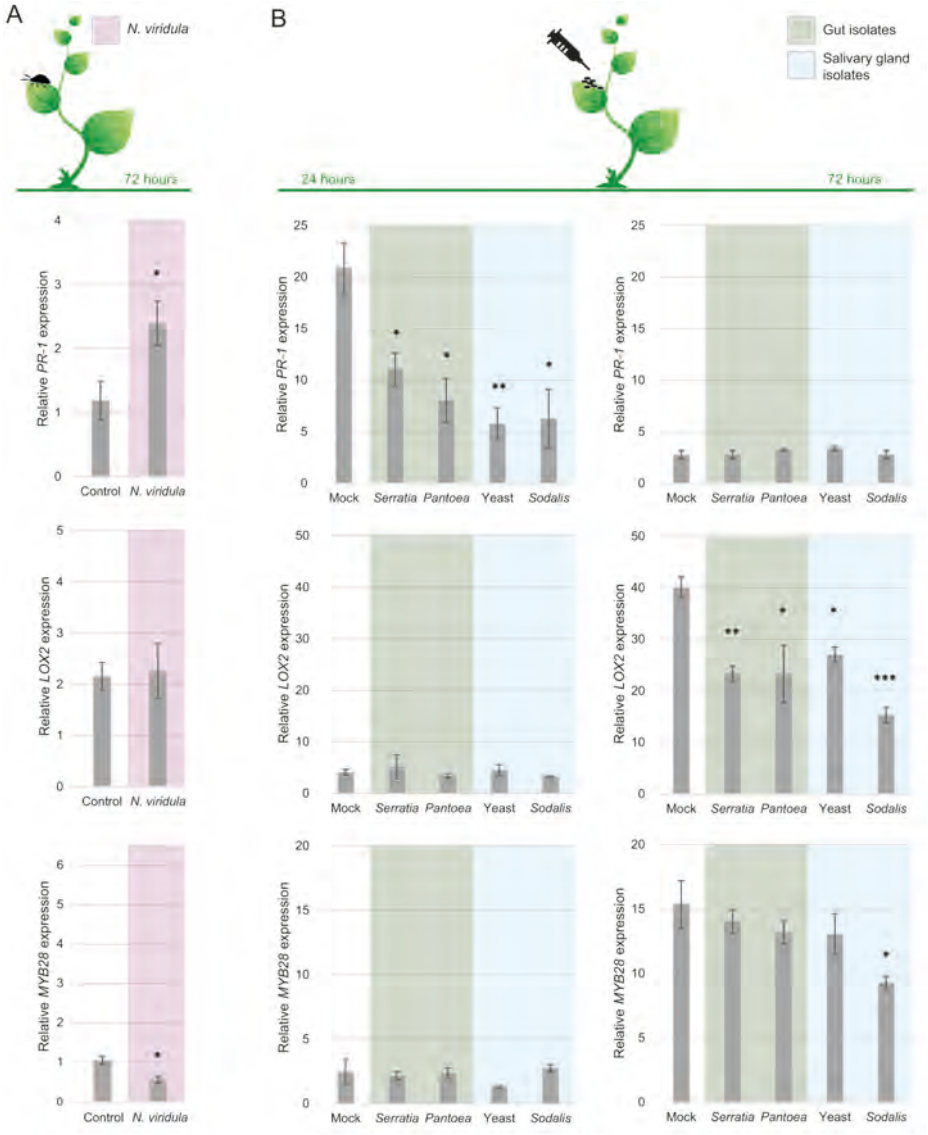

Figure 3.7. Plant defence repression by *N. viridula*-associated microorganisms. A) *PR-1*, *LOX2,* and *MYB28* gene expression of local leaves (feeding damage) from 5-week-old *A. thaliana* plants infested by *N. viridula* adults for 72 hours and control plants without *N. viridula* ($n = 3$, 6 plants, 9 technical replicates). **B)** *PR-1*, *LOX2,* and *MYB28* gene expression of 5-week-old *A. thaliana* plants at 24 and 72 hours after pierce-inoculation with a 5 µL droplet of 10 mM MgSO4 (*i.e.*, mock) or 1x10^8 CFU/mL of either *Serratia*, *Pantoea*, *Yeast* or *Sodalis* culture suspension in 10 mM MgSO4 ($n = 3$, 3 plants, 4 technical replicates). Bars represent the average gene expression relative to *PP2AA3* and error bars represent the standard error of means. Asterisks show Student's t-test significant differences between mock and treatment (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

Discussion

N. viridula is a notorious pest insect threatening global food production (Todd, 1989). Different pest management strategies were shown ineffective, due to *N. viridula*'s fast adaptive abilities, resulting in the proposition of an integrated approach in which a variety of preventive and therapeutic methods are used (Botías et al., 2019; Costa et al., 2007; Knight & Gurr, 2007; Palumbo et al., 2016). Since most insects rely on symbiotic microbiota for essential tasks, pest management strategies specifically targeting insect microbiota may provide an additional facet in integrated pest management for *N. viridula*. Strikingly, *N. viridula* microbiota is still poorly investigated, leaving untapped opportunities. To this end, we characterized *N. viridula* microbiota throughout their development as well as for their functional potential.

Our study revealed that the core microbiota of *N. viridula* predominantly consisted of *Pantoea*, *Sodalis, Serratia*, and *Commensalibacter* and to a lesser extent other genera such as *Klebsiella* and *Pseudomonas*, accounting for more than 99% of all microbial members. We determined that *Pantoea* and *Sodalis* were vertically transmitted via eggs and thus most likely function as obligate symbionts (Geerinck et al., 2022; Perlmutter & Bordenstein, 2020). *Pantoea* was previously described in Pentatomidae such as *N. viridula* and the elimination resulted in severe fitness defects of the insect host, confirming its obligate symbiotic nature (Hosokawa et al., 2016). *Sodalis* was associated with Pentatomidae before (Hosokawa et al., 2015), and we were able to pinpoint its predominant localization and highly abundant colonization of salivary glands. Moreover, with metagenomic analysis we shed light onto the role and host-specific adaptations and of *N. viridula* core microbiota, such as genome reduction of *Pantoea* and *Commensalibacter* (Akman et al., 2002; Karamipour et al., 2016; Shigenobu et al., 2000), presence of T3SS in *Sodalis*, which could allow cellular invasion and modulation of plant defences (Dale et al., 2002; Egan et al., 2014), and ability of microbiota to biosynthesize amino acids and B vitamin, degrade plant carbohydrates and toxic secondary plant metabolites. Although, *Pantoea* and *Sodalis* seem to be most dominant *N. viridula* symbionts, lower abundant *Serratia, Klebsiella, Commensalibacter, Pseudomonas,* and *Cutibacterium* could also benefit its insect host. *Serratia* was previously described as a facultative symbiont of insects that could be obtained via plants, and in this study we demonstrated its possible participation in detoxifying symbiosis via culturing it with toxic NPA (Pons et al., 2019). Even though some *Serratia* species are notorious plant disease-causing organisms that colonize

the plant's phloem (Bruton et al., 2003) no visual disease symptoms were observed in our plant inoculation experiments. Further, *Klebsiella* was detected in symbiotic relations with insects including Mediterranean fruit flies (*Ceratitis capitata*) and showed competitive capacities to pathogenic host gut inhabitants (Behar et al., 2005; Ciolfi & Marri, 2020). *Commensalibacter*, isolated from the midgut of *Drosophila*, has been demonstrated to protect against gut pathogens and our finding additionally implies its contribution to degradation of α-solanine and α-chaconine (Ryu et al., 2008). Also, *Pseudomonas* sp. Nvir isolated from *N. viridula* gut could detoxify NPA and therefore possibly benefits its host (Rogowska-van der Molen et al., 2022). Besides bacterial symbionts, we also isolated two yeast species *Wickerhamia* and *Candida* sp. from salivary glands and although insect-associated yeasts are still poorly studied, Matsuura et al. (2018) suggested their nutritional support to insects by biosynthesizing amino acids and vitamins.

Along with nutritious, protecting and detoxifying properties of *N. viridula*-associated microbes, we found that *N. viridula* transmits bacteria during feeding via saliva which altered plant defences. *N. viridula* induced SA (*i.e. PR-1*) plant defences, which is in line with previously published results (Giacometti et al., 2016), whereas all isolated microorganisms repressed SA defences early after inoculation. *N. viridula* repressed the aliphatic glucosinolate pathway (*i.e., MYB28*) involved in defence against insects and *Sodalis*, predominantly located in the salivary glands, was the only microorganism significantly suppressing the latter pathway. This observation along with the metagenomic evidence of T3SS presence further strengthens the hypothesis that *Sodalis* is a key player in repressing the biosynthesis of secondary plant metabolites. Altogether, *N. viridula* microorganisms seem to have a secret agenda in supporting their host with detoxifying and suppressing properties. However, to fully verify that *N. viridula* microbiota mediates detoxification and plant defence repression, eliminating *N. viridula* microbiome would provide the required evidence for microbial involvement in these processes. Nonetheless, to this day eliminating all microbiota remains challenging, since *N. viridula* requires its microbiota for viability and development (Capuzzo et al., 2005; Ceja-Navarro et al., 2015; Tada et al., 2011).

In conclusion, our study reveals the developmental and organ-specific dynamics of *N. viridula* core microbiota, revealing the important roles of *Pantoea*, and *Sodalis* as obligate symbionts and *Serratia* and *Commensalibacter* as facultative symbionts. *N. viridula* symbionts were found to be involved in host plant defence

repression and *Serratia* revealed *in vitro* detoxifying activities on toxic crown vetch metabolite NPA. Our results show the importance of studying tri-trophic interactions between insects, associated microbiota, and host plants to obtain fundamental knowledge on interactions between different organisms that could lead to the development of sustainable pest management strategies.

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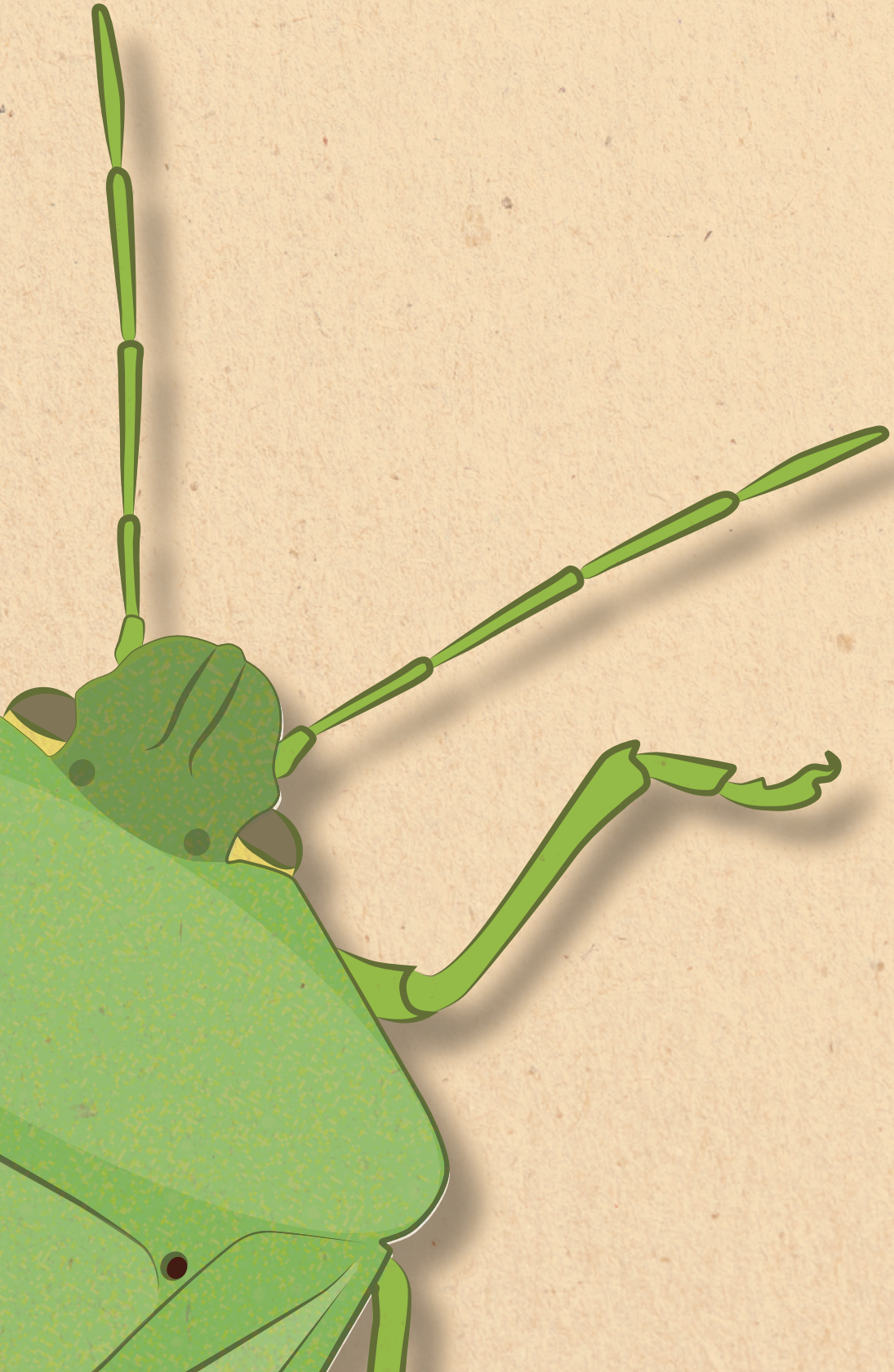

Chapter 4

From eggs to guts: Obligate association of *Sodalis nezarae* sp. nov. with the Southern green shield bug *Nezara viridula*

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Abstract

Phytophagous insects commonly engage in symbiotic relationships with bacteria that contribute to digestion, nutrient supplementation, development and detoxification for the hosts. The gut microbiota of Pentatomidae shield bugs was shown to be mainly located in the midgut and predominantly colonized by *Pantoea* symbionts, which benefit insects by providing essential nutrients. However, other members of the shield bug microbiota have been largely overlooked. Here we investigated the microbiota of the Southern green shield bug *Nezara viridula*, a piercing and sucking pest insect, and discovered an obligate *Sodalis* symbiont (Nvir). Confocal microscopy revealed the presence of *Sodalis* in multiple *N. viridula* organs, with a notable prevalence in salivary glands, and anterior regions of the midgut. The spatial distribution of the symbiont in organs was possibly linked to its functions leading to our hypothesis that *Sodalis* likely participates in repressing plant defences, digestion and nutrient supplementation. The removal of the external egg microbiota via surface sterilization had a profound impact on insect viability and led to retarded growth. However, it did not disrupt the vertical transmission of *Sodalis* and *Pantoea* symbionts, indicating the presence of internal microbiota associated with eggs. Next, the presence of *Sodalis* and *Pantoea* in sterilized eggs was confirmed by diagnostic PCR. Based on the dominance of *Sodalis* in testes, we deducted that *N. viridula* males could be involved in the vertical transmission of symbionts. Genomic analyses comparing various *Sodalis* species revealed that *Sodalis* sp. Nvir shared characteristics with the free-living *S. praecaptivus* and the *obligate* insect-associated *S. pierantonii* strain SOPE. Strain Nvir also displayed genome instability typical of some endosymbiont lineages, which suggested ongoing speciation from its once free-living ancestor to an obligate endosymbiont. On the basis of the apparent obligate nature of *Sodalis,* we propose that this symbiont of *N. viridula* be classified as a representative of a novel species, *Sodalis nezarae* sp. nov. (type strain Nvir). Altogether, the knowledge of symbiotic microbiota associated with *N. viridula* will contribute to the elucidation of targeted pest control strategies in the future.

Introduction

Insects establish symbiotic relationships with microorganisms which can confer important physiological traits to their host. This way, microbes allow insects to adopt new lifestyles which may enable them to colonize diverse plant species (Dillon & Dillon, 2004; van den Bosch & Welte, 2017). Particularly plant-feeding true bugs (Hemiptera) rely on their symbionts for digestion, nutrition and defence against pesticides (Colman et al., 2012; Feng et al., 2019; Kikuchi, Hayatsu, et al., 2012; Salem et al., 2014). Within the Hemiptera order, shield bugs (Pentatomidae) represent the largest family, including 8000 species of which several are crop pests (McPherson et al., 2017). One such insect is *Nezara viridula*, also known as the Southern green shield bug, a polyphagous species that feeds on over 30 plant families including crops (*e.g.* tomato, soybean, wheat), thereby causing major economic losses worldwide (McPherson & McPherson, 2000). Current pest control methods rely on insecticides, even though they were found to be largely ineffective against shield bugs. Recent studies, however, highlighted the potential of targeting symbiotic microorganisms as an alternative pest control strategy (Chung et al., 2018; Gonella & Alma, 2023). Microbiota associated with *N. viridula* was shown to aid the insects in overcoming plant defences by deactivating protease inhibitors and degrading soybean isoflavonoids, and the leguminous toxin 3-nitropropionic acid (NPA) (Medina et al., 2018; Rogowska-van der Molen et al., 2022; Zavala et al., 2015). Although *N. viridula* microbiota has shown to be beneficial to the host (Medina et al., 2018; Prado et al., 2009; Tada et al., 2011), the composition and function of insect microbiota outside of egg have until recently remained poorly characterized (Geerinck et al., 2022). A better understanding of the host-microbe relationship in *N. viridula* could eventually contribute to the development of sustainable and targeted pest control (Gonella & Alma, 2023; Rogowska-van der Molen et al., 2023).

Many of the gut symbionts are essential for the viability of their shield bug hosts. For example, experimental removal of the *N. viridula* egg microbiota with surface sterilization disrupted nymphal infection with symbionts and severely increased nymphal mortality (Tada et al., 2011). Several studies have described the co-evolution of plant-feeding true bugs with their symbionts by the development of specialized organs to house and sustain a stable symbiotic population. In Pentatomidae insects, the primarily obligate *Pantoea* symbionts are typically harboured in crypts of the posterior M4 region of the gut (Bistolas et al., 2014; Duron & Noel, 2016; Hosokawa et al., 2016; Kikuchi, Hosokawa,

et al., 2012; Matsuura et al., 2012; Taylor et al., 2014). Next to *Pantoea* located in the M4 crypts, it has been suggested that shield bugs harbour *Sodalis* as a core symbiont in anterior gut compartments (Fourie et al., 2023). Recently, a number of studies have reported the occurrence of *Sodalis* sp. bacteria in various insects and characterized them as obligate and facultative symbionts. Obligate *Sodalis* symbiont of slender pigeon louse *Columbicola columbae* was found to be maternally transmitted in bacteriocytes, contributing to the host insect by supplementing nutrients and participating in digestion (Fukatsu et al., 2007). Moreover, several co-obligate *Sodalis*-like symbionts have been identified in aphids (Manzano-Marín et al., 2023; Manzano-Marin et al., 2017) and Garber et al. (2021) described the recent acquisition of intracellular *Sodalis* endosymbionts in mealybugs. Also, *Candidatus* Sodalis baculum sp. nov associated with the bacteriome heteropteran seed bug *Henestaris halophilus* was reported to have an important role in complementing its host diet (Santos-Garcia et al., 2017). On the other hand, studies have reported that *Sodalis*-allied bacteria can function as facultative symbionts in acorn weevils, psyllids and shield bugs, although their symbiotic role remains unclear (Ghosh et al., 2020; Kaiwa et al., 2010; Toju & Fukatsu, 2011). While some authors suggested a secondary role for *Sodalis* in shield bugs, a recent study on *N. viridula* showed a high abundance of *Sodalis* and *Pantoea* in the egg microbiome, indicating their primary role (Geerinck et al., 2022).

As most research on shield bugs has focused on the obligate *Pantoea* symbionts and on M4 crypts, bacterial species like *Sodalis* have possibly been missed. Nothing is currently known about the abundance of *N. viridula*-associated bacteria in organs other than the gut. This knowledge could be crucial to understanding the symbiont transmission to the offspring and eventually be used in pest control. In this study, we removed symbionts in an egg surface-sterilization experiment and used 16S rRNA gene amplicon sequencing to determine the vertical transmission of *Sodalis* and *Pantoea*. The presence of bacteria in *N. viridula* organs was visualized with confocal microscopy with a specific focus on *Sodalis*. The results indicated a high prevalence of the symbiont in salivary glands, testes and anterior regions of the midgut which is possibly linked to its beneficial role in *N. viridula*. Finally, a comparative genomic analysis unveiled the divergent nature of the *Sodalis* symbiont of *N. viridula* which displays typical of an endosymbiotic lifestyle marked by vertical transmission. These findings provided valuable insights into the insect-microbe symbiosis field and broadened our understanding of *Sodalis* spp. as insect-associated bacteria.

Materials and Methods

Insect collection and rearing

Nezara viridula shield bugs were collected in the field on creeping thistle (*Cirsium arvense*) in the Netherlands (51.348028, 6.128802) in July 2019, and were provided by Wageningen Plant Research (Bleiswijk, the Netherlands) in March 2023. The insects were transferred to a greenhouse and placed in a rearing cage to establish a colony. *N. viridula* individuals were reared in a greenhouse facility at room temperature with normal daylight and additional light to obtain a photoperiod of 16:8 hour (light:dark) year-round. Insects were provided with sunflower (*Helianthus annuus*), soybean (*Glycine max*), brown mustard (*Brassica juncea*) seeds, flat beans (*Phaseolus vulgaris*), and the native plants crown vetch (*Securigera varia*), black mustard (*Brassica nigra*) and black nightshade (*Solanum nigrum*).

Egg sterilization

Six freshly laid egg masses were collected from the *N. viridula* population. Three masses were surface sterilized by submerging in 1 mL 70% ethanol for 1 min. As a control, three egg masses were treated with 1 mL autoclaved demineralized water for 1 min. For detection of *Sodalis* and *Pantoea* symbionts in and outside of eggs, one sterilized and one control egg mass was crushed using a sterile pestle after which DNA was isolated and used for diagnostic PCR.

To assess the influence of microbiota removal on the survival and composition of *N. viridula* microbiota, two sterilized and two control egg masses were placed in separate rearing cages at room temperature (photoperiod 16:8 h, light:dark) containing sunflower, soybean, brown mustard seeds and flat beans. The survival rate was monitored by tracking the number of insects until reaching adulthood. To analyse the gut microbial community, three adult insects were randomly selected from each cage. The individuals were dissected, and DNA was extracted from their gut systems. The gut microbiota composition was determined using 16S gene rRNA amplicon sequencing.

Insect dissection

To determine the effect of surface sterilization on microbiota composition, complete gut systems (M1-M4 sections and hindgut) of adult *N. viridula* were dissected directly after submersion of insects in 70% ethanol for approximately one min after which movement stopped. Dissection was performed under non-sterile conditions using a stereomicroscope, scalpel, and forceps. Separation

of tissues from the insect body was performed in phosphate-saline buffer (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4) to prevent tissue rupture. Gut systems were disrupted by vigorously pipetting and vortexing in 100 µL PBS for DNA isolation.

DNA isolation

Isolation of the DNA for 16S rRNA gene amplicon sequencing and diagnostic PCR was performed with a DNeasy PowerSoil kit (QIAGEN, the Netherlands). Disrupted tissue, eggs and plant pellets were directly transferred into the lysis buffer in the Powerbead tubes and tubes were vortexed for 10 min at 50 Hz using a TissueLyser LT (QIAGEN). DNA was eluted in 40 µL Nuclease Free Water (Thermo Fisher Scientific Inc., Waltham, USA) and quantified with a Qubit dsDNA HS assay kit (Thermo Fisher Scientific Inc.). Before PCR, DNA concentrations of organ samples had been standardized to the equal concentration of 2 ng μL^{-1}.

16S rRNA gene amplicon sequencing and analysis

The gut bacterial community of *N. viridula* was determined by amplification of the V3-V4 region of the 16S rRNA gene. The sequencing was performed by Macrogen (the Netherlands) with *Bac341F* and *Bac806R* primers (Caporaso et al., 2011; Herlemann et al., 2011) using an Illumina MiSeq sequencer (Illumina; Supporting Information Table S2). Paired-end (2x 301bp) reads libraries were prepared with the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina).

The quality of the raw paired-end sequences was checked with FastQC 0.11.8 (Bushnell, 2014). The reads were then filtered, and adapters were trimmed. Approximately 40,000-70,000 paired-end sequencing reads were obtained per sample. The data were further processed using the DADA2 1.8 pipeline (Callahan et al., 2016) in R. Phylogenetic taxonomy of the reads was assigned using the SILVA 16S rRNA gene database 138.1 (Quast et al., 2012). Count data were normalized to relative abundance. Microbial community profiles were analysed and amplicon sequence variants (ASVs) were visualized using the phyloseq (McMurdie & Holmes, 2013) and ggplot2 (Wickham, 2009) packages in R.

Phylogenetic inference

For phylogenetic placement of the previously isolated *Sodalis* sp. Nvir (Coolen, Rogowska-van der Molen et al. *under review*), we collected genomes of a comprehensive dataset of *Pectobacteriaceae*, "*Bruguierivoracaceae*" (Li et al., 2021), and four outgroups, totalling 65 bacterial strains. Next, OrthoFinder v2.5.4 (Emms & Kelly, 2019) was used to group predicted protein-coding genes

across selected genomes in families of orthologous proteins. Then, we aligned all single-copy marker genes (i.e. present across all genomes) using MAFFT v7.490 (-maxiterate 1000 -localpair; (Katoh & Standley, 2013)), concatenated them, and then subjected the alignment to Gblocks v0.91b (Talavera & Castresana, 2007) for removal of divergent and ambiguously aligned blocks. Phylogenetic inference was done using IQtree v2.2.2.7 (LG4X+I+G; (Minh et al., 2020)) with 1000 UltraFast bootstrap replicates (Hoang et al., 2018). The resulting tree was visualised and exported for editing using Figtree v1.4.4 (https://github.com/rambaut/figtree).

ANI and 16S rRNA gene identity scoring

To calculate pairwise ANI scores, we extracted the genomic sequences for selected relatives of *Sodalis* sp. Nvir. These sequences were used as input for OthoANIu v1.2 (Lee et al., 2016). For guidance, species and genus thresholds were set following Chun et al. (2018) and Barco et al. (2020).

For calculating 16S rRNA gene identity, selected 16S rRNA gene sequences were extracted and aligned using the built-in Muscle v3.8.425 (Edgar, 2021) from AliView v1.28 (Larsson, 2014). Last, Clustal Omega v1.2.4 (Sievers et al., 2011) was used to calculate a pairwise 16S rRNA gene identity matrix. The species and genus level threshold were set following Kim et al. (2014) and Yarza et al. (2014).

Phage region identification

To infer prophage regions across the selected *Sodalis* genomes, we extracted the nucleotide sequences for all genes per organism. These were then used as input for PHASTEST (Arndt et al., 2016; Wishart et al., 2023; Zhou et al., 2011). Visualisation and export of diagrams were done using the web-server tool.

Diagnostic PCR

The presence of *Sodalis* and *Pantoea* symbionts in sterilized and control egg masses was determined with a PCR reaction quantifying *gro*L gene using specific primers *SodF* (5′- CCCTTATCGATAGCCGCGTT-3′) and *SodR* (5′-GATCTTCATTGTCGCCACGC-3′) and *PantF* (5′-TCGCAGTTCTAAAGGTGGGT-3′) and *PantR* (5′-GTAGCGCCACTTTAATGCCA-3′), respectively (Coolen, Rogowska-van der Molen et al. *under review*). Additionally, *Sodalis* presence in organs of adult *N. viridula* male and female was determined using the same *Sodalis*-specific primer pair. As a positive control, DNA from a *Sodalis* isolate obtained from *N. viridula* salivary glands (Coolen, Rogowska-van der Molen et al. *under review*) was used, whereas Nuclease Free Water (Thermo Fisher Scientific Inc.) served

as a negative control. Amplification was performed using SensoQuest labcycler (BIOKÉ, the Netherlands) in a 25 mL reaction volume and consisted of 12.5 µL PerfeCTa Quanta SYBR Green FastMix (Quanta Bio, USA), forward and reverse primers of the final concentration of 0.1 pmol μL^{-1}, 1 µL of template DNA and 9.5 µL Nuclease Free Water (Thermo Fisher Scientific). The PCR programme was initiated by initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, elongation at 72°C for 30 s, and 5 min at 72°C for the final elongation. PCR products were examined by electrophoresis at 80 V for 1 hour in a 1.5% (w/v) agarose gel containing ethidium bromide in 1 x SB buffer. To determine PCR product size GeneRuler 100 bp DNA ladder was used as a marker DNA (Thermo Fisher Scientific). The electrophoresis gel was examined in UV light.

Fluorescence *in situ* hybridization

To image total bacteria, Gammaproteobacteria and the *Sodalis*, we dissected the fat body, ovary, testes, salivary glands, M1, M2, M3, M4 sections of the midgut, hindgut and Malpighian tubules from one adult male and one female *N. viridula* as described under *Insect dissection*. Isolated organs were washed twice with PBS and incubated with 300 µL PBS and 900 µL paraformaldehyde solution (4% paraformaldehyde in PBS) for 2 hours on ice and samples were washed twice with 500 µL PBS. The fixed tissue samples were placed on SuperFrost Plus™ adhesion microscope slides (Epredia™, USA) and dehydrated in increasing ethanol concentration (50%, 80%, 100%) for 10 min each. Then the samples were air-dried and incubated for 3 hours at 46°C with a hybridization buffer (0.9 M NaCl, 0.02M Tris-HCl (pH 8.0), 35% formamide, 0.02% sodium dodecyl sulfate) and specific probes in the humidified chamber. For total bacterial detection and identification of specific bacterial groups within the samples, fluorescence *in situ* hybridization (FISH) using a Fluos-labelled general bacterial probe (Eub-mix; (Amann et al., 1990; Daims et al., 1999)) and a Cy5-labeled Gammaproteobacterial probe (GAM42A; (Manz et al., 1992)) along with a GAM42A competitor probe were employed. Along, a Cy3-labeled probe specific for *Sodalis* sp. was used for targeted detection (Sod1238R; (Koga et al., 2013)). The final concentration of Cy3 and Cy5-probes in the hybridization buffer was 0.5 pmol μL^{-1} (Sod1238R and GAM42A) and a Fluos-probe 0.83 pmol μL^{-1} (Eub-mix).

After hybridization, tissue samples were washed in the wash buffer (0.2 M Tris-HCl (pH 8), 0.08 M NaCl, 5 mM EDTA) for 10 min at 48°C, dipped in ice-chilled ultrapure Milli-Q® water, air-dried and embedded in Vectashield without

4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Images were taken using a confocal laser scanning microscope (Leica SP8X, Mannheim, Germany) and image processing was performed in Fiji (Schindelin et al., 2012).

Results

Egg-surface sterilization decreases *N. viridula* survival

Symbionts are instrumental to the development and fitness of *N. viridula.* However, upon the recent discovery of an internal egg microbiota (Geerinck et al., 2022; Tada et al., 2011) we questioned how the removal of symbionts from the egg surface impacts *N. viridula* survival rate as well as symbiont acquisition (Figure 4.1). We observed that upon symbiont removal from the egg surface, the survival rate of *N. viridula* decreased to 11% while the control population reached 86% (Figure 4.1A). Moreover, the removal of symbionts negatively affected the appearance of adult females (Figure 4.1B) and resulted in discolouration and retarded growth. To assess the effect on the microbiome, we applied 16S rRNA gene amplicon sequencing, which revealed that the gut microbial community of control non-sterilized insects was dominated by *Pantoea* and *Sodalis* (Figure 4.1C). These genera were also highly abundant in sterilized insects, but the abundance of *Sodalis* was significantly lower in sterilized insects compared to the control population (45% vs 20%, Student's *t*-test; *P*-value <0.05). In addition, in two sterilized insects, the relative abundance of *Sodalis* decreased in favour of *Enterobacter* and *Enterococcus*, suggesting that egg-surface sterilization allowed for colonization of the gut by bacteria from the surrounding environment. Altogether, these results demonstrated that the removal of symbionts from the egg surface had an adverse effect on insect survival and external appearance, while the two main symbionts *Pantoea* and *Sodalis* were still dominating the gut microbial community. This raised the question of whether those bacteria might be transmitted inside the eggshell.

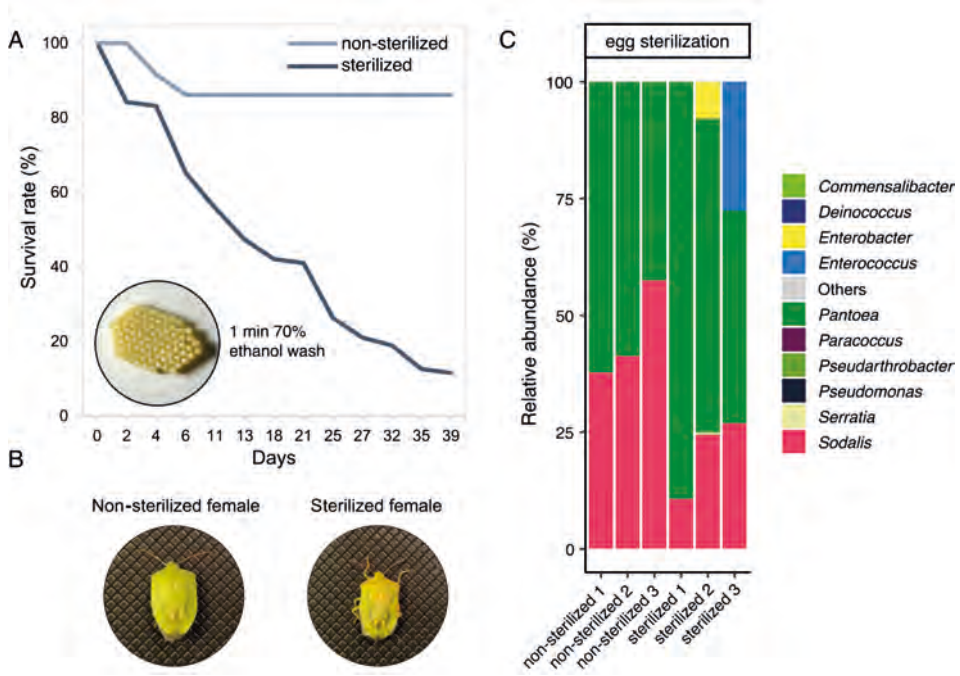

Figure 4.1. The effect of egg-surface sterilization on survival and gut microbial community of *Nezara viridula*. A) Survival rate was monitored from hatching until reaching adulthood in non-sterilized (control) and egg-surface sterilized populations of *N. viridula*. Eggs in a sterilized population (n = 95) were treated with a 1 min wash in 70% ethanol while non-sterilized eggs (n = 89) were subjected to 1 min wash in demineralized autoclaved water. **B)** External appearance of the control and treated adult females. **C)** Gut microbial community composition in adult *N. viridula* individuals based on 16S rRNA gene amplicon sequencing subjected to symbiont removal via egg-surface sterilization or non-sterilized. The taxonomy is displayed at the genus level. 'Others' represent the amplicon sequence variants (ASVs) that average below 0.5% of all reads. Individual bar graphs represent the sequencing of one adult insect per bar, with three biological replicates (n = 3) per treatment group.

Sodalis dominates the microbial community in salivary glands and the anterior section of the midgut

N. viridula and other Pentatomidae insects are commonly associated with obligate *Pantoea* symbionts, located in the M4 crypts, however little is known regarding their other symbiotic partners. Here, we investigated the prevalence of the obligate symbiont *Sodalis* in organs and eggs. Hatching shield bug nymphs acquire symbionts that colonize the gut tract via ingestion of maternally smeared symbionts from the egg surface (Figure 4.2A) (Hosokawa et al., 2016), so surface-sterilization of egg clusters probably results in a disturbance in their acquisition. We compared surface-sterilized to surface-washed egg clusters of

N. viridula using diagnostic PCR; this confirmed the presence of *Pantoea* and *Sodalis* in non-sterilized eggs, and also detected DNA of *Pantoea* and *Sodalis* symbionts in surface-sterilized egg cluster, corroborating the recently reported internal egg microbiome (Geerinck et al., 2022).

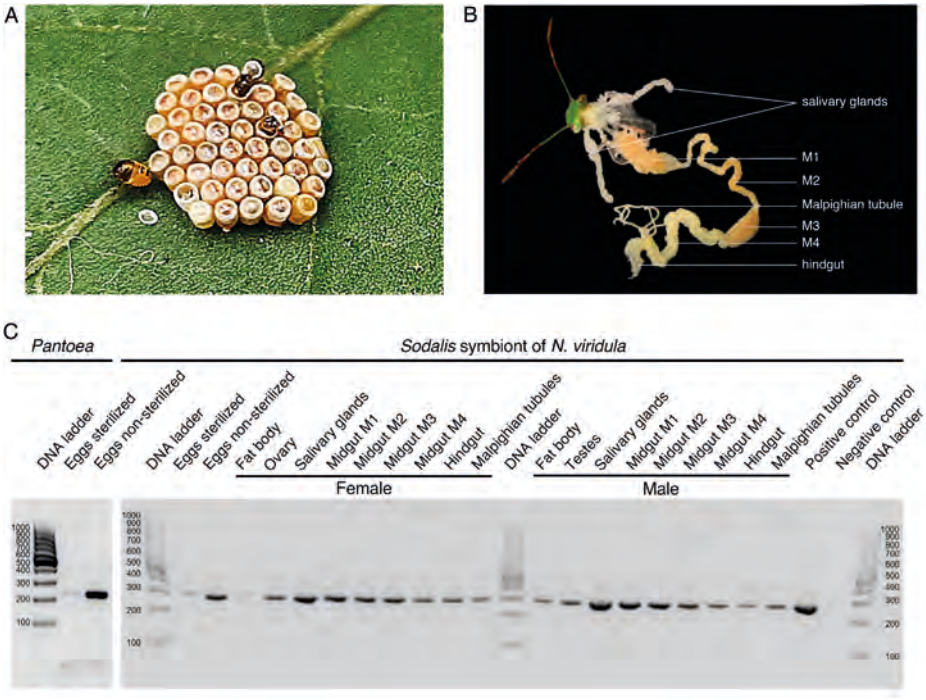

Figure 4.2. *Sodalis* distribution in *Nezara viridula*. A) Hatching of nymphs from the egg of *N. viridula*. **B)** Organization of organs dissected from adult *N. viridula*. M1, midgut first section; M2, midgut second section; M3, midgut third section; M4, midgut fourth section with crypts. **C)** Diagnostic PCR detection of obligate *Pantoea* and *Sodalis* symbionts in non-sterilized and surface-sterilized eggs and *Sodalis* in organs of female and male adult *N. viridula*. Negative control was nuclease-free water while positive control was the DNA extracted from the isolated *Sodalis* strain from the *N. viridula* salivary glands. DNA extracted from eggs, organs and tissue was normalized to an equal concentration of 2 ng μL^{-1}.

Next, we sought to investigate the localization of the *Sodalis* symbiont in organs of *N. viridula* (Figure 4.2B-C). Diagnostic PCR analysis of dissected tissues detected the symbiont DNA in all *N. viridula* organs with the most prominent band in salivary glands. Notably, *Sodalis* was present throughout the entire gut system, but it seems to prefer colonization of anterior segments of the midgut. Additionally, *Sodalis* was observed in reproductive organs, as well as in the Malpighian tubules.

To verify the localization of *N. viridula*-associated microbiota and the *Sodalis* symbiont, we performed fluorescence *in situ* hybridization (FISH) on organs and visualized them with confocal laser scanning microscopy. Our analysis revealed that bacteria formed clusters in the entire principal salivary gland (Figure 3A-B). The tissue was colonized with Gammaproteobacteria, of which *Sodalis* was a dominant member. The gut of shield bugs is a complex alimentary system colonized with symbionts and is divided into a midgut that consists of four sections (M1-M4), and a hindgut (Figure 4.2B) (Tada et al., 2011). Confocal microscopy of the gut system indicated the presence of primarily Gammaproteobacteria in all gut sections, and *Sodalis* in M1 and M3 (Figure 4.4). In addition, M1 seemed to harbour more *Sodalis* than M3, whereas no *Sodalis* was found in the M2 and M4 sections or the hindgut. The M4 section was densely colonized with Gammaproteobacteria other than *Sodalis* which were largely localised to the surrounding crypts (Figure 4.4D). Taking the PCR and FISH imaging observations together, we demonstrated that a *Sodalis* symbiont is present in various *N. viridula* digestive organs.

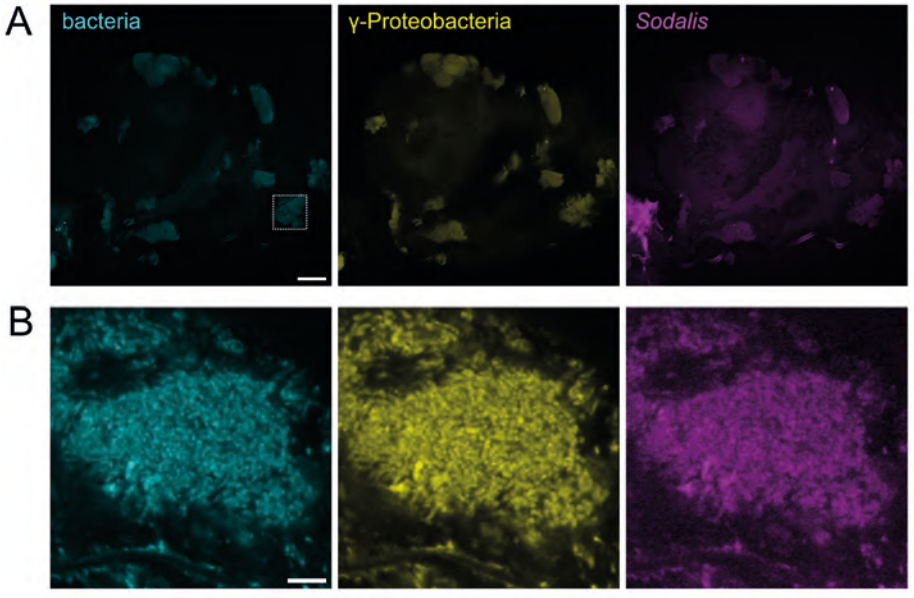

Figure 4.3. Colonization of *Nezara viridula* salivary glands by Gammaproteobacteria and *Sodalis*. Confocal laser scanning micrographs show the adult *N. viridula* salivary gland where bacteria are visualized with fluorescence in situ hybridization. **A)** Overview micrographs of a principal salivary gland. A dashed square in the left panel marks the localization of the magnified panel in B) Scale bar = 100 µm. Left panel, detection of all bacteria with probe EUB-mix in cyan (Fluos); middle panel, γ-proteobacteria detected with probe GAM42A in yellow (Cy5); right panel, *Sodalis* detected with probe Sod1238R in magenta (Cy3) **B)** Magnified confocal micrographs indicated in panel A) with a dashed square of the principal salivary gland. Scale bar = 5 µm.

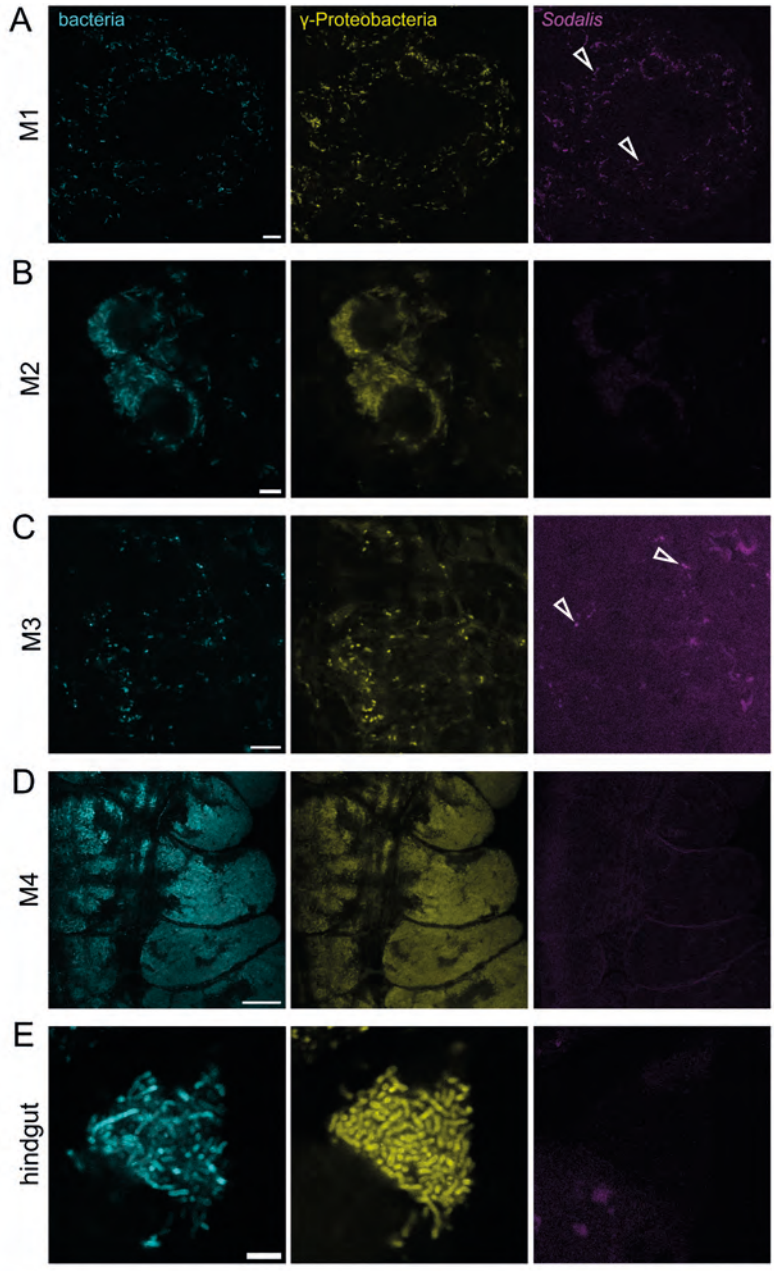

Figure 4.4. Colonization of the *Nezara viridula* gut by Gammaproteobacteria and *Sodalis*. Confocal micrographs show the adult *N. viridula* intestinal tract. Panel bacteria shows FISH-probe Eub-mix in cyan (Fluos). Panel γ-Proteobacteria shows FISH-probe GAM42A in yellow (Cy5). Panel *Sodalis* shows FISH-probe Sod1238R in magenta (Cy3). Open arrowhead points at FISH-stained Sodalis. **A)** M1 section of the gut. Scale bar = 10 µm. **B)** M2 section of the gut. Scale bar = 10 µm. **C)** M3 section of the gut. Scale bar = 10 µm. **D)** M4 section of the gut with crypts. Scale bar = 25 µm. **E)** Hindgut. Scale bar = 5 µm.

The presence of *Sodalis* in testes suggests paternal symbiont transmission

According to the diagnostic PCR analysis, *Sodalis* DNA was present in reproductive organs and Malpighian tubules, so we aimed to visually confirm these findings. Confocal microscopy of the reproductive organs in which microorganisms were visualized with FISH indicated a lack of microorganisms in the ovary (Supplementary Figure 1A). This finding corroborates the recently reported absence of microbes from dissected ovaries and unlaid eggs (Geerinck et al., 2022; Kikuchi et al., 2009). Furthermore, our results revealed a dense population of Gammaproteobacteria and *Sodalis* in the testes (Figure 4.5A) suggesting a possible paternal involvement in symbiont transmission. Besides this, Malpighian tubules were colonized with Gammaproteobacteria (Figure 4.5B), and only a few microbes were seen in the fat body (Supplementary Figure 1B). Since those microbes were localized on the tissue surface, they were unlikely to be part of the fat body microbiome and rather represent bacteria that were part of the gut microbiome. Taken together, we showed the symbiont distribution in *N. viridula* organs and provided the first evidence of the possible participation of shield bug males in the vertical transmission of symbionts.

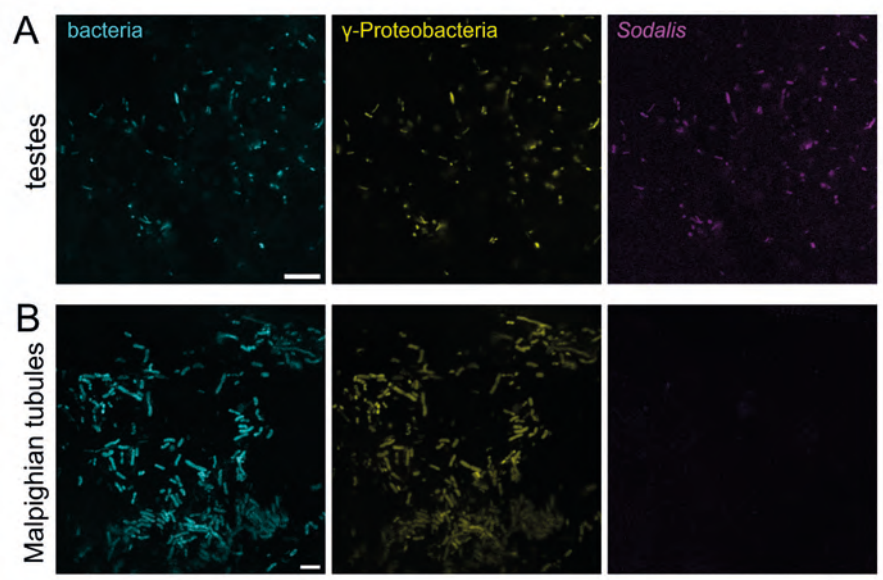

Figure 4.5. Colonization of *Nezara viridula* testes and Malpighian tubules by Gammaproteobacteria and *Sodalis*. Confocal micrographs show the adult *N. viridula* testes A) and B) Malpighian tubules. Panel bacteria shows FISH-probe Eub-mix in cyan (Fluos). Panel γ-Proteobacteria shows FISH-probe GAM42A in yellow (Cy5). Panel *Sodalis* shows FISH-probe Sod1238R in magenta (Cy3). **A)** Testes. Scale bar = 10 µm. **B)** Malpighian tubules. Scale bar = 5 µm.

Phylogenetic placement and genomic characteristics indicate that *Sodalis* sp. Nvir is a novel symbiont

Although several authors suggested the facultative role of *Sodalis* symbionts associated with other shield bug species (Kaiwa et al., 2010, 2011; Matsuura et al., 2014), the high abundance of *Sodalis* sp. Nvir in organs and eggs of *N. viridula* and a vertical transmission route implied an obligate rather than facultative nature (Ferrari & Vavre, 2011). To unveil the role of *Sodalis* sp. Nvir we characterized its genome and compared it to the non-host associated and endosymbiotic *Sodalis* strains. Using single-copy core genes of representatives from the *Pectobacteriaceae* and "*Bruguierivoracaceae*" (Li et al., 2021), we recovered *Sodalis* sp. Nvir as sister to *S. praecaptivus* strain HS1 (Figure 4.6A and Supplementary Figure S2). The 16S rRNA gene identity values of *Sodalis* sp. Nvir and its close relatives (98.57-99.22%) suggested that *S. praecaptivus*, *S. pierantonii*, *S. melophagi*, and strains TME1 and Nvir all belonged to the same species (Supplementary Table S1; (Kim et al., 2014)). However, the Average Nucleotide Identity (*ANI*) values of the pairwise comparisons between *S. glossinidius* strain morsitans and the aforementioned strains fell below the recommended values for the species rank (Chun et al., 2018) but well above those for the genus (Barco et al., 2020). These values suggested that *S. glossinidius* has likely recently diverged from the *S. melophagi* +*S. pierantonii* +*S. praecaptivus* clade, keeping a high sequence identity of its 16S rRNA gene but a more divergent genome sequence.

When comparing the *Sodalis* sp. Nvir with its closest relatives with a high-quality genome available, it became evident that this novel symbiont found itself in an intermediate stage between the free-living *S. praecaptivus* and the obligate host-associated *S. pierantonii* strain SOPE (Figure 4.6B). While all strains maintained a similar GC content, the genome size of *S. pierantonii* strain SOPE displayed an inferred reduction of about 1 Megabase pair when compared to *S. praecaptivus* strain HS1 and strain Nvir. Additionally, the colonisation by numerous prophages of the two insect-associated symbionts (strains SOPE and Nvir) was evident, displaying 21 and 20 such elements, with several predicted to be inactive due to lack of completeness. In addition, strain Nvir displayed a large number of plasmids (six) when compared to HS1 (one) and SOPE (none), which is a feature of genome instability displayed by some endosymbiont lineages (Campbell et al., 2017; Campbell et al., 2015; Van Leuven et al., 2014). Moreover, at least one of those plasmids likely was or originated from, a prophage (SoNvir_pl05), and three had their origins in the HS1 chromosome or a related molecule (SoNvir_pl01, pl02, and pl06), as suggested by a blastn search *vs.* HS1. These

results suggested a divergent strain Nvir that is undergoing speciation from its once free-living ancestor, as supported by the genome characterization, FISH imaging of *N. viridula* organs and vertical transmission route. Given the tradition and convenience of giving different specific names to insect host-associated *Sodalis* endosymbionts that are vertically transmitted and obligate for their hosts, we proposed that *Sodalis* sp. Nvir represents a novel species within the *Sodalis* genus: *Sodalis nezarae* sp. nov., with strain Nvir being the sole sequenced representative of this taxon.

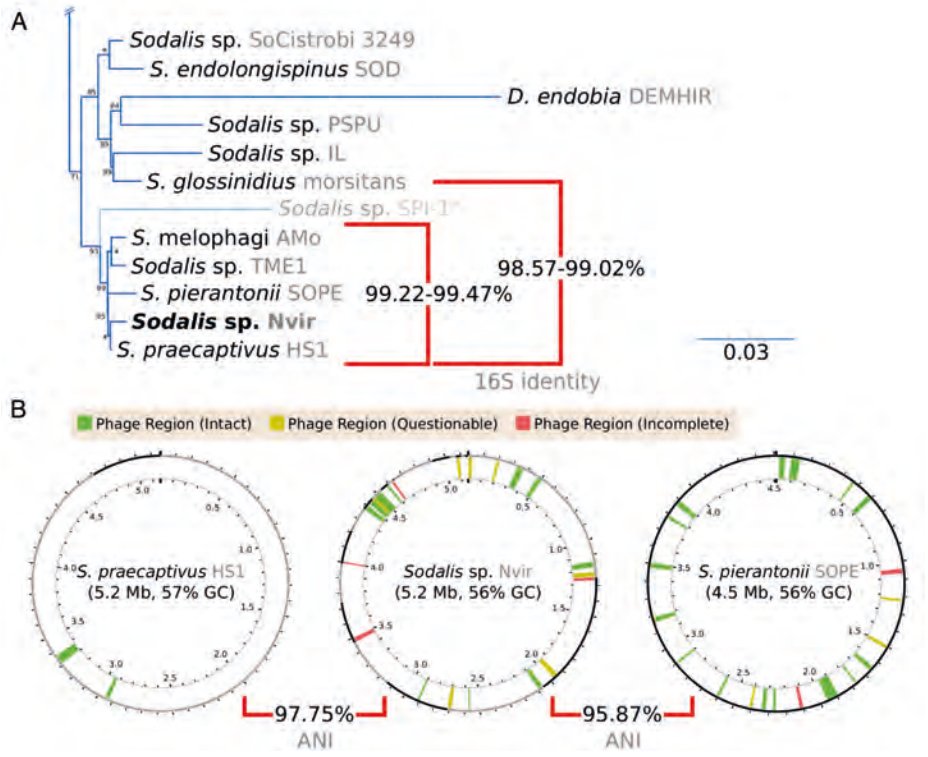

Figure 4.6. Phylogenetic placement and genomic features of *Sodalis* sp. Nvir. A) Excerpt of Maximum-Likelihood phylogenetic placement of the novel *Sodalis* sp. Nvir within the *Sodalis* clade. Names on leaves specify the bacterial genera, species, and strains (in grey). Red squared brackets denote a two-way comparison of 16S rRNA gene identity values. Values at nodes indicate the UltraFast bootstrap support values in percentages. An asterisk (*) denotes a support of 100%. *Sodalis* sp. SPI-1 was excluded for the visual grouping representing 16S rRNA gene identity value scores. **B)** Circular diagrams of selected *Sodalis* genomes. Numbers at inner ticks denote the location within the genome in Mega base pairs. The outer ring represents contigs/molecules of the genome assembly, with alternate grey and black colours separating them. Phage regions are coloured as specified in the colour key at the top of the diagrams. Squared brackets indicate the two-way ANI values.

'*Candidatus* Sodalis nezarae' sp. nov.

We propose the specific name '*Candidatus* Sodalis nezarae' for the obligate *Sodalis* endosymbiont of the Southern green shield bug *Nezara viridula*. This symbiont colonized salivary glands, anterior regions of the midgut and testes, as confirmed by diagnostic PCR, using *Sodalis*-specific primers *SodF* (5'-CCCTTATCGATAGCCGCGTT-3') and *SodR* (5'-GATCTTCATTGTCGCCACGC-3') and FISH microscopy performed on dissected tissues. Indirect evidence for its vertical transmission came from the presence of this symbiont in the testes as well as the egg surface and interior. The type strain, Nvir^{T}, was isolated from salivary glands of *Nezara viridula* collected in 2019 feeding on host plants (sunflower soybean, brown mustard seeds, flat beans, crown vetch, black mustard and black nightshade). The genome size was 5,497,650 bp and the G+C content of the type strain was 55.6 mol%. The draft genome sequence of this strain has been deposited in the GenBank database under the Project number PRJEB70466 and submission ERA27452063. A previous assembly of this symbiont from a metagenomic sequencing effort of *N. viridula* can be found under accession number CAUIKD000000000.

Discussion

N. viridula is a piercing and sucking insect that feeds on nutritionally imbalanced phloem sap, thus it relies on symbiotic associations with bacteria to biosynthesize essential nutrients (Tada et al., 2011). Pentatomidae shield bugs are associated with obligate *Pantoea*-like symbionts which colonize crypts in the M4 section of the gut (Duron & Noel, 2016), but little is known about other members of the shield bug microbial community. Hosokawa et al. (2015) suggested that *Sodalis* symbionts are facultatively associated with most shield bugs, however, recent studies proposed an obligate nature of *Sodalis* symbionts in *N. viridula* due to its evident benefit for the host and a vertical transmission route. *Sodalis* dominates the external egg-associated microbial community and, together with *Pantoea*, was highly abundant inside egg shells (Geerinck et al., 2022). This suggests that *Sodalis* is important to the host and most likely fulfils essential symbiotic functions to *N. viridula*. Along with that, the insect was shown to transmit *Sodalis* via saliva to plants, which repressed the biosynthesis of secondary plant metabolites allowing the insect to cope with plant defences (Coolen, Rogowska-van der Molen et al. *under review*).

In this study, we comprehensively characterized the prevalence of the previously overlooked *Sodalis* symbiont of *N. viridula* in eggs, insect tissues and organs. Our findings revealed that *Sodalis* constituted an integral component of both the external and internal egg microbiome, confirming previous reports by Geerinck et al. (2022). The authors compared egg-associated microbiomes from *N. viridula* eggs obtained from two distinct geographical locations. Although they used a more stringent protocol involving 70% ethanol and 1.5% sodium hypochlorite to remove external egg microbiota in comparison to this study, their data revealed that the Belgian population had two primary egg-associated symbionts, *Pantoea* and *Sodalis.* In their study, *Sodalis* exceeded 70% of the relative abundance on the egg surface and 20% inside eggs. Surprisingly, *Sodalis* was not found to be a part of the internal or external egg-associated microbiome in the Italian population. These variations, however, might be attributed to the difference in the insect genotype as previously reported in other distinct populations of *N. viridula* (Geerinck et al., 2022; Hosokawa et al., 2016; Medina et al., 2018).

The eggs of the Pentatomidae family species are typically covered with a protective layer which is secreted by the females during oviposition (Shan et al., 2021). It forms a physical barrier for the developing nymphs preventing dehydration and pathogen entrance as well as protects symbionts and secures their transmission to offspring. Prado et al. (2009) and Tada et al. (2011) found that surface sterilisation drastically decreased *N. viridula* vitality and development. However, our observations indicate that despite egg-surface sterilization, *Sodalis* and *Pantoea* were acquired in the adult *N. viridula* microbiome as evidenced by their presence in the microbial community profiles of the intestinal tract. This implies a likely origin of symbionts from the inside of the egg. Also, Prado et al. (2006) reported that up to 60% of *N. viridula* nymphs hatched from surface-sterilized eggs acquired symbionts, however, no symbionts were successively transferred to the next generation. Nevertheless, the removal of symbionts from egg shells negatively impacted *N. viridula* survival and appearance. Surface sterilization decreased the abundance of *Sodalis* and *Pantoea*, and thus could result in less efficient colonization of the host and a decreased survival rate. Moreover, it led to the acquisition of *Enterobacter* and *Enterococcus* with simultanous decrease in the relative abundance of *Sodalis*, suggesting potential repression of *Sodalis* growth by environmental microbes. The disturbance of the egg microbiota might have contributed to the easier invasion of entomopathogenic bacteria, which has adverse effects on insect fitness and survival rate (Tozlu et al., 2019). Studies have shown, that shield bugs

have a susceptible time window during the 2nd instar period for the acquisition of symbionts, including those which are horizontally transmitted, explaining the presence of *Enterococcus* and *Enterobacter* in sterilized populations (Kikuchi et al., 2011). Although the efficiency of surface sterilization was not analysed in this study and others have used various methods to remove the external egg microbiome, our results demonstrate that other factors such as the environmental conditions and genetic traits could influence the performance and dynamics of shield bug populations. Furthermore, our findings strongly suggest the ability of *N. viridula* to retain *Sodalis* and *Pantoea* in the event of external egg microbiome disturbance and maintain vertical transmission of microbes via internal storage of symbionts in eggs. Taken togther, this underlines the complexity behind the manipulation of the egg microbiome, symbiont acquisition and its effect on insect fitness, and therefore should be considered in the context of the pest insect management strategies.

Through our investigation, we determined that the *Sodalis* symbiont is present in the majority of *N. viridula* organs, but predominant in the salivary glands, testes, and anterior regions of the midgut. *Sodalis* symbionts were first reported in tsetse flies but have since been observed in weevils, psyllids, aphids, mealybugs, and various shield bugs (Ghosh et al., 2020; Hosokawa et al., 2015; Kaiwa et al., 2011; Koga & Moran, 2014). Although *Sodalis* infections are rare in most shield bug species, a high abundance of symbiont in *N. viridula* indicates a significant selective pressure favouring its presence (Hosokawa et al., 2015; Kaiwa et al., 2010, 2011). Also, the structural organization of symbionts in specific organs is possibly controlled by the host. In honey bees, the colonization of microbes is orchestrated by the insect which secretes organic acids into the gut lumen favouring the growth of symbiotic *Snodgrassella alvi* (Quinn et al., 2024). On the other hand, Kim et al. (2013) described that insect midgut epithelia could produce antimicrobial substances and, in that way, control the selective infection of symbionts to the M4 midgut crypts. Moreover, the recent discovery of the sorting organ, a constricted region between the M3 and M4 section of the midgut, underscores its pivotal role in facilitating the shield bug gut symbiosis (Ohbayashi et al., 2015). Although it is unclear whether a comparable organ exists in *N. viridula*, a similar pattern was observed in the decreasing abundance of *Sodalis* along the intestinal tract. The analysis showed that *Sodalis* colonized anterior parts of the gut, including the M3 region, and no *Sodalis* was observed during imaging of the posterior M4 region including the crypts suggesting the presence of a sorting mechanism in *N. viridula*. The structural arrangement of *Sodalis* in specific organs is possibly linked to its function for the host.

Hence, the colonization of salivary glands may be linked to *Sodalis'* ability to repress plant defences, while participation in digestion, detoxification, and nutrient supplementation could be associated with gut colonization (Coolen, Rogowska-van der Molen et al. *under review*). Interestingly, the presence of Gammaproteobacteria and *Sodalis* in the testes and the absence in ovaries and unlaid eggs collected from the ovaries as shown before (Geerinck et al., 2022; Kikuchi et al., 2009), suggests that *N. viridula* males play a role in vertical symbiont transmission to the offspring. Tsetse flies were shown to maternally and paternally transmit obligate intracellular *Wolbachia* symbiont, illustrated by the detection of microbes in ovaries and testes (Doudoumis et al., 2012). Likewise, *Sodalis glossinidius* was shown to be transmitted from males to females during mating (De Vooght et al., 2015) and Watanabe et al. (2014) discovered that bacterial symbiont *Rickettsia* is vertically transmitted via sperm in leafhopper *Nephotettix cincticeps*. Also, paternal transmission of bacteria was observed in *Anopheles stephensi* mosquitos (Damiani et al., 2008). However, to date, there has been limited focus on the male reproductive organs within the Pentatomidae family. Whether parental transmission of symbionts via internal egg microbiome occurs in *N. viridula* and other shield bugs is of interest and deserves future studies.

Genomic studies on *Sodalis* revealed that the genus includes three lineages of which two appear to be associated with a symbiotic lifestyle (*Ca*. S. pierantonius and *S. glossinidius*) and one represents a non-host-restricted lineage and therefore could serve as a valuable reference of the ancestral state to infer *Sodalis* genome evolution (Clayton et al., 2012; Lo et al., 2016). This particular lineage, *S. praecaptivus*, was described as an opportunistic human pathogen (Oakeson et al., 2014). Interestingly, unlike other insect-associated microbes, a phylogenomic analysis showed no evidence of co-speciation events among *Sodalis* symbionts (Hosokawa et al., 2006; Kikuchi et al., 2009; Renoz et al., 2023), which raised questions regarding *Sodalis* acquisition by insects. Our comparative genomic analysis of various *Sodalis* species revealed that *Sodalis* sp. Nvir shared characteristics with the free-living *S. praecaptivus* and the obligate host-associated *S. pierantonii* strain SOPE. *Sodalis* sp. Nvir displayed a large number of plasmids that suggested genome instability and adaptation towards a symbiotic lifestyle (Campbell et al., 2017; Van Leuven et al., 2014). Also, obligate mealybug-associated *Sodalis* species have been reported to harbour a relatively large genome which contained thousands of pseudogenes, indicating its recent shift from a free-living to an endosymbiotic lifestyle (Garber et al., 2021). In the context of insect symbiosis, *Sodalis* probably fulfils diverse

functions for the insect host contributing to nutrient provisioning, reproductive fitness and defence against pathogens (Manzano-Marín et al., 2023; Manzano-Marin et al., 2017; Renoz et al., 2023; Sloan & Ligoxygakis, 2017). While the metabolic potential of *Sodalis* sp. Nvir requires further investigation, our findings point to the divergent nature of *Sodalis* sp. Nvir and showed the strain's evolutionary journey from once free-living ancestor to an obligate *N. viridula* symbiont.

In conclusion, our research demonstrated the vertical transmission of symbionts through the shield bug's unique ability to harbour *Sodalis* and *Pantoea* within its eggs. Our findings suggest a potential role of males in this transmission process, as illustrated by the presence of bacteria within the testes microbiome. Besides this, the study provided evidence for selective control in the gut colonization orchestrated by *N. viridula* and revealed that previously overlooked *Sodalis* is an obligate symbiont that undergoes genome reorganization as an adaptation to symbiotic life. Altogether, our results present an example of the intimate relationship between insects and microbes which could be essential in the development of targeted pest control strategies in the future.

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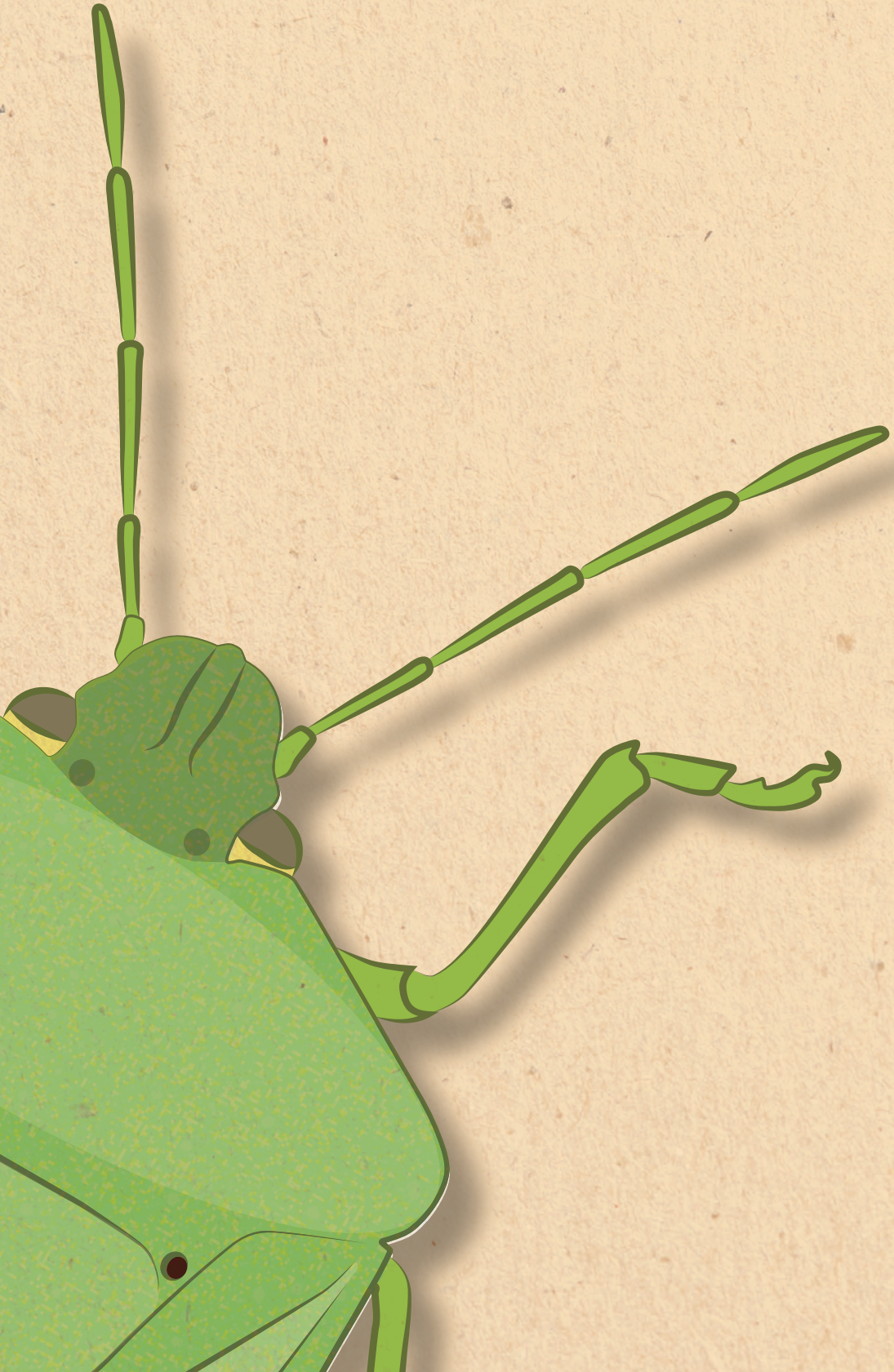

Chapter 5

Comparative genomics of insect-associated *Commensalibacter* reveals host-specific adaptations

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Abstract

Acetic acid bacteria are ubiquitous microbes in sugar-rich environments like fruits, flowers, and nectar. The family of acetic acid bacteria includes 19 different genera, of which *Commensalibacter* is the least studied. *Commensalibacter* has been associated with insects feeding on sugars, such as honeybees, bumble bees, and butterflies, in which it likely provides health benefits to the hosts. Nevertheless, little is known about *Commensalibacter* phylogeny and its role in insects. In this study, we performed comparative genomics on six *Commensalibacter* genomes obtained from isolates or metagenomes from the common fruit fly, honeybee, monarch butterfly, and the Southern green shield bug. Phylogenomic analyses revealed that the six *Commensalibacter* strains comprised four species and that strains obtained from the same host cluster together. The genomes shared 1227 protein-coding sequences and showed a similar metabolic potential for energy conservation. Each strain also contained numerous strain-specific genes that indicated host-specific adaptations towards carbohydrate metabolism and amino acid and B vitamin biosynthesis. A detailed analysis of the accessory genome of a novel *Commensalibacter* strain (Nvir) originating from the Southern green shield bug indicated its potential to detoxify the toxic secondary plant metabolites α-solanine and α-chaconine, which could benefit the host by supporting resistance to plant defences. Altogether, we elucidate the metabolic potential within the genus *Commensalibacter* and reveal genomic specialisation towards a host-specific symbiotic lifestyle.

Introduction

Acetic acid bacteria (AAB) are ubiquitous, obligate aerobic, Gram-negative *Acetobacteraceae* present in carbohydrate-rich and acidic environments, and alcoholic beverages (Bartowsky & Henschke, 2008; Crotti et al., 2010; Saichana et al., 2015). They are known for their unique metabolism, oxidative fermentation, which is a process of incomplete oxidation of alcohols and carbohydrates that results in the release of aldehydes, ketones, and organic acids (He et al., 2022). For this reason, AAB are commercially exploited in the industrial production of cellulose, gluconic acid, acetic acid, and vinegar (Embuscado et al., 1994; Gullo et al., 2014; Kersters, 2006).

AAB are widespread in sugar-rich environments like fruits, flowers, nectar, and fermented foods. They are symbiotically associated with insects since several *Acetobacteraceae* were isolated from the intestinal tracts of honeybees (*Apis mellifera*), pink sugarcane mealybugs (*Saccharicoccus sacchari*), common fruit flies (*Drosophila melanogaster*), and mosquitos (*Anopheles gambiae*) (Favia et al., 2007; Roh et al., 2008; Ryu et al., 2008; Servin-Garciduenas et al., 2014; Siozios et al., 2019). Symbiotic bacteria can be beneficial to insects since they biosynthesize essential nutrients allowing their host insects to feed on nutrient-deficient food sources, such as nectar and plant sap (Dillon & Dillon, 2004). Besides this, symbionts are involved in digestion, repression of opportunistic pathogens, and detoxification of plant chemical defence compounds (Coolen et al., 2022; van den Bosch & Welte, 2017). Like other symbionts, some AAB adapted to living in symbiotic relationships with insects. For example, Chouaia et al. (2014) applied genomic analysis to two insect isolates *Asaia platycodi* and *Saccharibacter* sp. to show that AAB have functional traits for adaptation to symbiotic life, explaining their prevalence in insects. The study showed that AAB harboured several secretion systems and transporters which could allow crosstalk between symbiont and host. Furthermore, AAB biosynthesize polysaccharides like cellulose, which allow them to adhere to insect cells and colonize host tissues to maintain an intimate interaction with their host (Barak et al., 2007; Crotti et al., 2010). As a result, AAB have been found to localize to various insect body compartments. For instance, *Asaia* resides in the gut epithelium, salivary glands, and reproductive systems of mosquitos. Its presence in reproductive systems might be relevant to the transmission of AAB to offspring (Crotti et al., 2009). Yet, despite these common associations of AAB with insects, AAB may not always be essential to their hosts, since antibiotic removal of AAB from mosquitos did not affect the insect's survival (Favia et al., 2007).

As of 2021, 19 bacterial genera are classified as AAB, including *Acetobacter*, *Asaia*, *Bombella*, *Commensalibacter, Gluconacetobacter*, and *Kozakia* (Qiu et al., 2021). *Commensalibacter* is one of the insect-associated AABs and is a core member of the gut bacterial community of honeybees, monarch butterflies, and common fruit flies (Botero et al., 2023). *Commensalibacter* has been associated with host health and protection against opportunistic pathogens: *Commensalibacter intestine* A911^{T} was able to modulate fruit fly immunity by suppressing pathogenic *Gluconobacter morbifer* (Ryu et al., 2008). Likewise, *Commensalibacter* might participate in digestion, since its increasing relative abundance in the Welwitschia bug (*Probergrothius angolensis*) across developmental stages was linked with different nutritional requirements of the bug (Martinez et al., 2019). Nevertheless, *Commensalibacter* is still poorly studied, and little is known about its origin, functional diversity, and role in insect life.

To further understand the role of *Commensalibacter* in insect life and to unveil its metabolic potential and adaptations toward a symbiotic lifestyle, we performed a comparative genomic analysis on *Commensalibacter* genomes associated with insects. We collected five publicly available genomes of *Commensalibacter* from honeybees, monarch butterflies, and common fruit flies and compared them to the metagenome of *Commensalibacter* sp. Nvir that we collected from the Southern green shield bug (*Nezara viridula*) gut microbiome. Our analysis revealed that the *Commensalibacter* genus shares a large core genome but that each strain harbours strain-specific genes indicating host-specific adaptations towards carbohydrate metabolism, and amino acid and B vitamin biosynthesis. Moreover, the novel *Commensalibacter* sp. Nvir strain showed the metabolic potential to degrade toxic secondary plant metabolites and, thus, could support *N. viridula's* resistance to plant defences.

Materials & Methods

Insect rearing

Nezara viridula (42 insects, 2nd -4th instar nymphs) were collected in the field from creeping thistle (*Cirsium arvense*) in the Netherlands (51.348028, 6.128802) on the 5th of July 2019. The insects were transferred to a greenhouse and placed within a rearing cage to establish a colony. *N. viridula* individuals were reared in a greenhouse facility with normal daylight and additional light to obtain a photoperiod of 16:8h (light: dark) year-round. Insects were provided

with sunflower (*Helianthus annuus*), soybean (*Glycine max*), and brown mustard (*Brassica juncea*) seeds and native crown vetch (*Securigera varia*), black mustard (*Brassica nigra*) and black nightshade (*Solanum nigrum*) plants.

Insect dissection and DNA isolation

Five complete gut systems (M1-M4 sections) of adult *N. viridula* were dissected, pooled, and mixed in an Eppendorf tube. Gut tissue was homogenized by brief vortexing in 200 µL of PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) with 2 mm glass beads. Subsequently, the samples were split, and equal parts were used for DNA extraction using the DNeasy PowerSoil kit and the DNeasy Blood & Tissue kit (QIAGEN) according to the manufacturer's instructions with modifications. Namely, 100 µL of gut or salivary gland suspension was bead-beaten on the TissueLyser LT for 2 min at 50 Hz and DNA was eluted with 50 µL Nuclease Free Water (Thermo Fisher Scientific Inc.). Eluted DNA was incubated at 65°C for 5 min and stored at 4°C until sequencing. Quantification of the extracted DNA was performed with a Qubit dsDNA HS assay kit (Thermo Fisher Scientific Inc.).

Genome sequencing, assembly, and annotation

Template DNA extracted from the guts and salivary glands of *N. viridula* was used to obtain the genome of *Commensalibacter* sp. Nvir. The DNA library was prepared with the Nextera XT Library Preparation Kit (Illumina, San Diego, California, United States) according to the manufacturer's instructions. The library was checked for quality and size distribution using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA kit (Agilent Technologies, Santa Clara, California, USA). Quantitation of the library was performed by the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc Waltham USA). Paired-end sequencing (2x300bp) was performed using the Illumina MiSeq sequencer (Illumina, San Diego, California, United States) and the MiSeq Reagent Kit v3 (Illumina) according to the manufacturer's protocol.

The quality of Illumina paired-end genomic sequencing data was assessed using FASTQC 0.11.8 (Bushnell, 2014) before and after quality processing. Quality trimming, adapter removal, and contaminant filtering of reads were performed using BBDuk (BBTools 38.75; Bushnell, 2014). Trimmed reads were co-assembled *de novo* using MEGAHIT 1.2.9 (Li et al., 2015). MEGAHIT assembled the genome using k-mer sizes of 21, 29, 39, 59, 79, 99, 119, and 141. Reads were mapped back to the genome using separately BBMAP 38.75 (default settings), Bowtie 2 2.3.5, or Burrows-Wheeler Aligner (BWA MEM) 0.1.17 (Bushnell, 2014;

Li & Durbin, 2010). The sequencing mapping files were handled and converted using SAMtools 1.10 (Li et al., 2009). Genome binning was performed for contigs greater than 1500 bp using four binning algorithms: BinSanity 0.3.1 (Graham et al., 2017), CONCOCT 1.1.0 (Alneberg et al., 2014), MaxBin2 2.2.7 (Wu et al., 2016), and MetaBAT 2 2.15 (Kang et al., 2019) using default settings. The bin was supplied to DAS Tool 1.1.2 (Sieber et al., 2018) for consensus binning to obtain the final bin. Taxonomic assignment for the trimmed sequencing reads and assembled genome was performed with Kaiju and CheckM (Menzel et al., 2016; Parks et al., 2015).

In addition, five publicly available *Commensalibacter* genome bins (Assemblies GCA_003691365.1 GCA_003202795.1, GCA_000527695.1, GCA_000231445.2, GCA_002152535.1) were downloaded from the NCBI database (March 2020) and compared with *Commensalibacter* sp. Nvir was obtained from *N. viridula*. The quality of all *Commensalibacter* bins was assessed through a single-copy marker gene analysis with CheckM 1.1.2 (Parks et al., 2015). The genomes were automatically annotated with Prokka 1.13.4 (Seemann, 2014). Genome annotations were examined using Artemis 16.0.0 (Carver et al., 2005), and, if necessary, manual annotation was performed. In the *Commensalibacter* sp. Nvir bin, two protein-coding sequences were elongated and in total 32 genes were added to the original annotated genome.

Phylogenomic analysis

The single-copy-core-gene-phylogeny-pipeline UBCG was used to determine the evolutionary relationship between the *Commensalibacter* strains which was visualised in Interactive Tree of Life (iTOL) (Letunic & Bork, 2021; Na et al., 2018). Furthermore, the Average Nucleotide and Amino Acid Identities (ANI and AAI) of the genome bins were determined using online tools from Kostas lab (Rodriguez-R & Konstantinidis, 2016).

Metabolic pathway analysis

Commensalibacter genome bins were individually mapped in the KEGG mapper using the KEGG Automatic Annotation Server (KAAS) to visualize the metabolic pathways (Kanehisa & Sato, 2020; Moriya et al., 2007). To establish the content of the core genome and the accessory genomes, orthologous clustering was performed using the web-based tool OrthoVenn2 with a cutoff value of an E-value of 10^{-15} and an inflation value of 1.5 (Xu et al., 2019). This information was used in combination with the output from KEGG for metabolic reconstruction and comparative analysis.

The annotation of genes visualized in the KEGG mapper was manually checked by comparing the annotation of PROKKA, KEGG, and OrthoVenn2, as well as the original annotations from the publicly available *Commensalibacter* assemblies. If the annotations conflicted with each other, bidirectional protein BLAST curation was done using the NCBI, InterPro, or UniProt databases (Coordinators, 2016; Mitchell et al., 2019; UniProt, 2021). If no closely related homologs could be found with BLAST, the program HMMER was used instead (hmmer.org version 3.3). The seed sequences were obtained from the Pfam database, TIGRFAMs database, or were generated after executing a multiple sequence alignment of the targeted protein from the UniProt database with Clustal Omega (El-Gebali et al., 2019; Madeira et al., 2019).

Protein function prediction was based on highly conserved amino acid sequences and was assessed by aligning protein-coding sequences (CDS) from the *Commensalibacter* strains with reference proteins using Clustal Omega (Sievers et al., 2011).

Results and Discussion

Commensalibacter has reduced genome size

To gain insight into host-specific adaptations of the insect-associated *Commensalibacter* genus, we performed comparative genomic analysis on publicly available genomes and compared it to the newly sequenced *Commensalibacter* sp. Nvir. The genome characteristics of six *Commensalibacter* genome bins isolated from the gut of *N. viridula*, *A. mellifera*, *D. plexippus,* and *D. melanogaster* are summarized in Table 5.1. All genomes were more than 95% complete and consisted of one strain as revealed by CheckM (<1% contamination). Also, all strains had a genome size varying between 1.96 and 2.45 Mbp, which is relatively small for AAB genomes that typically vary between 2.0 Mbp (*Bombella intestini)* and 4.5 Mbp (*Komagataeibacter intermedius*) (Dos Santos et al., 2015; Li et al., 2016). *Commensalibacter* also had a relatively low GC content of about 36.8-37.9%. The small genome sizes and low GC content are likely related to a symbiotic lifestyle since symbionts tend to have reduced genome sizes with increased AT content as a result of small population size, decreased recombination efficacy and constant nutrient environment (Van Leuven & McCutcheon, 2012).

Phylogenomic analysis revealed a new *Commensalibacter* species

To assess the evolutionary relationships and identify unique species, phylogenomic analyses were performed. Similarity clustering analysis of 92 single-copy marker genes revealed four separate lineages (Figure 5.1A). In combination with the ANI values and a 95% cutoff value (Figure 5.1B), the six *Commensalibacter* strains represent four *Commensalibacter* species. This finding supports an earlier report by Botero et al. (2023) in which four *Commensalibacter* species were identified in a phylogenomic analysis of 26 *Commensalibacter* isolates. Moreover, in the phylogenetic tree, strains obtained from the same host clustered together and shared 99% similarity in their ANI and AAI values with their counterpart (Figure 5.1B, C), thereby indicating diversification as a potential result of host-related speciation events (Goris et al., 2007; Richter & Rossello Mora, 2009). From an evolutionary perspective, the Nvir strain whose metagenome-assembled genome we analysed is the most distinct from other *Commensalibacter* strains.

Table 5.1. *Commensalibacter* genome characteristics included in the comparative genomic analysis.

Strain	Nvir	AMU001	ESL0284	MX01	A911^{T}	DmL 052
Host	*Nezara viridula*	*Apis mellifera*	*Apis mellifera*	*Danaus plexippus*	*Drosophila melanogaster*	*Drosophila melanogaster*
Chromosome size (Mbp)	2.01	2.01	1.96	2.31	2.45	2.43
GC content (%)	37.5	37.9	37.7	36.7	36.8	36.8
Completeness (%)	95.8	99.0	99.0	99.5	100	99.5
Contamination (%)	0	0.75	0.5	0.75	0.5	0.5
Protein (CDS)	1785	1768	1697	2077	2232	2205
Gene	1824	1832	1761	2125	2279	2249
Plasmid (Mb)	NA	0.01	NA	0.02 0.01	NA	NA
GC content (%)		31.3		28.8 32.2		
Genes		8		14 8		
Protein		8		12 7		
Contigs	145	2	1	12	26	50
Status	Contig	Complete	Complete	Contig	Contig	Contig

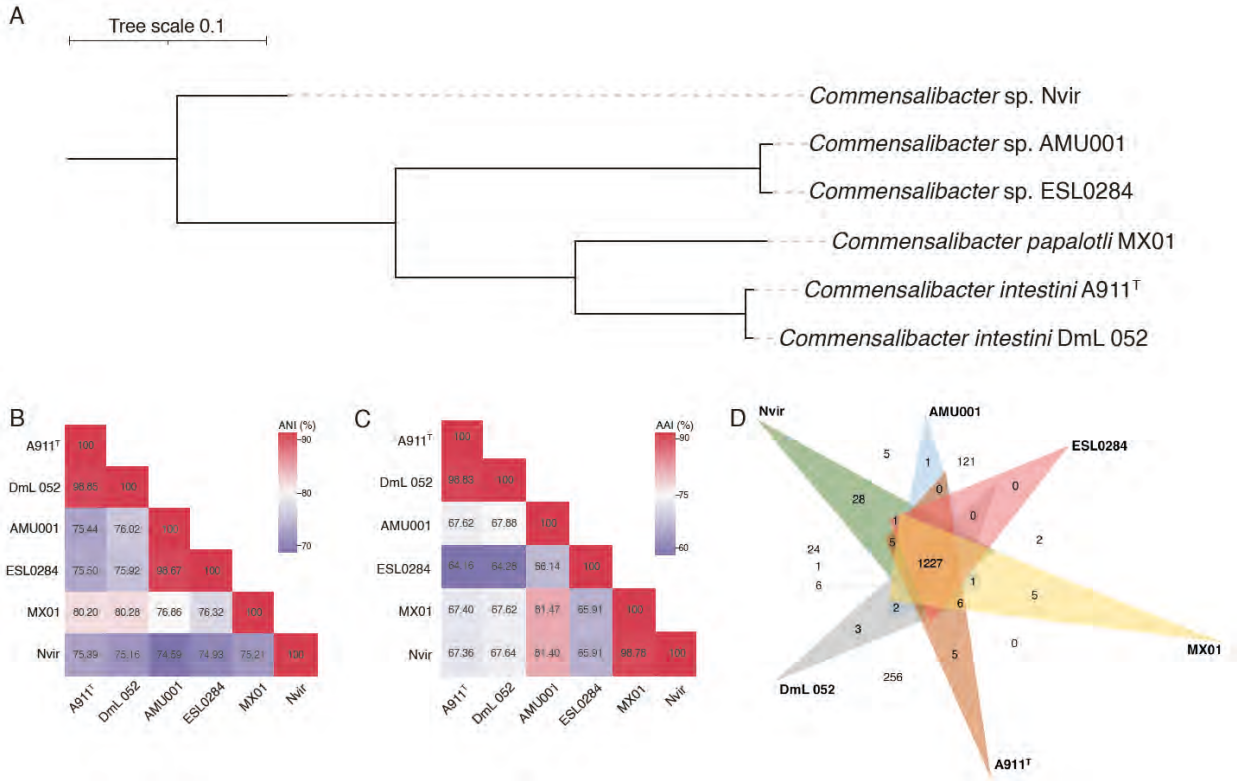

Figure 5.1. Genomic relationship between *Commensalibacter* strains. A) Maximum-likelihood phylogenetic tree based on the up-to-date bacterial core gene (UBCG) of 92 concatenated genes extracted from six *Commensalibacter* assemblies. The tree was reconstructed using iTOL. The heatmap of ANI **B)** and AAI **C)** values between the six *Commensalibacter* strains. The ANI/AAI matrix estimated all-vs-all distances between strains and clustered them based on their similarity. **D)** Orthologous clustering of protein-coding sequences of the *Commensalibacter* strains.

Core and host-related accessory genes show diversity within the *Commensalibacter* genus

Host-related genomic diversification of the *Commensalibacter* strains results in (1) a core set of genes that may include coding sequences (CDSs) important to the basic biological functions and genus identity of *Commensalibacter*, and (2) an accessory set of genes that may reveal host-specific adaptations. Ortholog identification analysis revealed that the strains we analyzed share 1227 CDS clusters (Figure 1D and Supplementary Table S1), of which 70% constitute the CDS pool in the Nvir, AMU001, and ESL0284 strains, and only 55-60% in the A911^{T}, DML 052 and MX01 strains. This finding corresponds with the phylogenomic analysis, which indicated a larger similarity between Nvir, AMU001 and ESL0284 strains than A911^{T}, DML 052 and MX01. Furthermore, the latter strains have a larger CDS cluster pool and encode 200 additional CDS clusters that are involved in a wide variety of metabolic processes (Supplementary Table S2). Interestingly, Nvir misses 223 CDS clusters that are shared between all other *Commensalibacter* species, which are involved mainly in the metabolism of vitamins and cofactors, suggesting that Nvir might contribute to supporting the insect's nutritional demands to a lesser degree.

In addition to the core genome, each *Commensalibacter* strain contained strain-specific CDS clusters. The analysis indicated that strain Nvir had the largest proportion of 28 unique CDS, whereas other strains had either none or only a few strain-specific CDS clusters, indicating the dissimilarity between *Commensalibacter* sp. Nvir and other *Commensalibacter* strains. Altogether, the differences between strains might be the result of strain-specific adaptations to differences in the environment, niche, host, and diet (Amato et al., 2019).

Central carbon metabolism analysis highlights host-related differences between *Commensalibacter* strains

Insect-associated *Commensalibacter* symbionts are still poorly investigated. Therefore to unveil the adaptations to the symbiotic lifestyle we analysed the metabolic potential of *Commensalibacter*. We compared the core genome as well as the accessory genome and analysed strain-specific genes to highlight differences between the strains.

AAB are adapted to sugar-rich environments like fruits, flowers, and plant sap by evolving to metabolize complex carbohydrates into simpler metabolites like D-glucose, D-galactose, glycerol, ethanol, and acetate. Based on the *Commensalibacter* core genome we reconstructed the central carbon metabolism

of the *Commensalibacter* species (Figure 5.2). Strain Nvir had the most extensive carbohydrate-degrading potential, especially for D-galactose, which might indicate species-dependent adaption to the host's diet. Nvir strain harbours a gene encoding for α-galactosidase-like MelA, which allows for the conversion of raffinose and stachyose to D-galactose (Silvestroni et al., 2002). Moreover, Nvir and MX01 both harbour the *lac* operon for lactose uptake and utilization (*lac*YZX) that enables the import and subsequent cleavage of the disaccharide lactose into D-glucose and D-galactose.

Unlike galactose/lactose metabolism, our analyses indicate that the glycolysis and pentose-phosphate pathway (PPP) are well conserved between *Commensalibacter* species, as is also shown in other AAB (Illeghems et al., 2013). Despite this conservation, all *Commensalibacter* genomes lack 6-phosphofructokinase, suggesting that the Embden-Meyerhof-Parnas pathway is only used for gluconeogenesis. For energy conservation, β-6-glucose-6P is oxidized with glucose-6-phosphate 1-dehydrogenase and 6-phosphoglucocolactonase to D-gluconate-6P in the Entner-Doudoroff pathway (reactions 3, 14, 15). Moreover, in contrast to the other *Commensalibacter* species, strain Nvir lacks a phosphoenolpyruvate carboxylase but may be using the ATP-dependent phosphoenolpyruvate carboxykinase instead.

On the other hand, phosphoenolpyruvate can be transformed via pyruvate kinase to pyruvate, which then can enter the TCA cycle via two routes. It can be actively converted to malate by an NADPH-dependent dehydrogenase, or it can be first converted to acetyl-CoA and CO_2 by a pyruvate-flavodoxin oxidoreductase. The pyruvate-flavodoxin oxidoreductase is present in all strains except Nvir, which likely reduces pyruvate via a formate C-acetyltransferase and yields formate instead of carbon dioxide. The sole reliance on formate C-acetyl transferase for the conversion of pyruvate to acetyl-CoA might not be however beneficial for Nvir as formate increases the acidic stress in the cell (Ribeiro et al., 2022). Strain Nvir and *Commensalibacter* strains that contain formate C-acetyltransferase could hence reduce the acidic stress induced by formate by activating a putative FocA-like formate transporter. This transporter regulates internal and external formate concentrations by a pH-dependent mode-switch that actively imports formate at low external pH and passively exports it at high external pH (Lu et al., 2011).

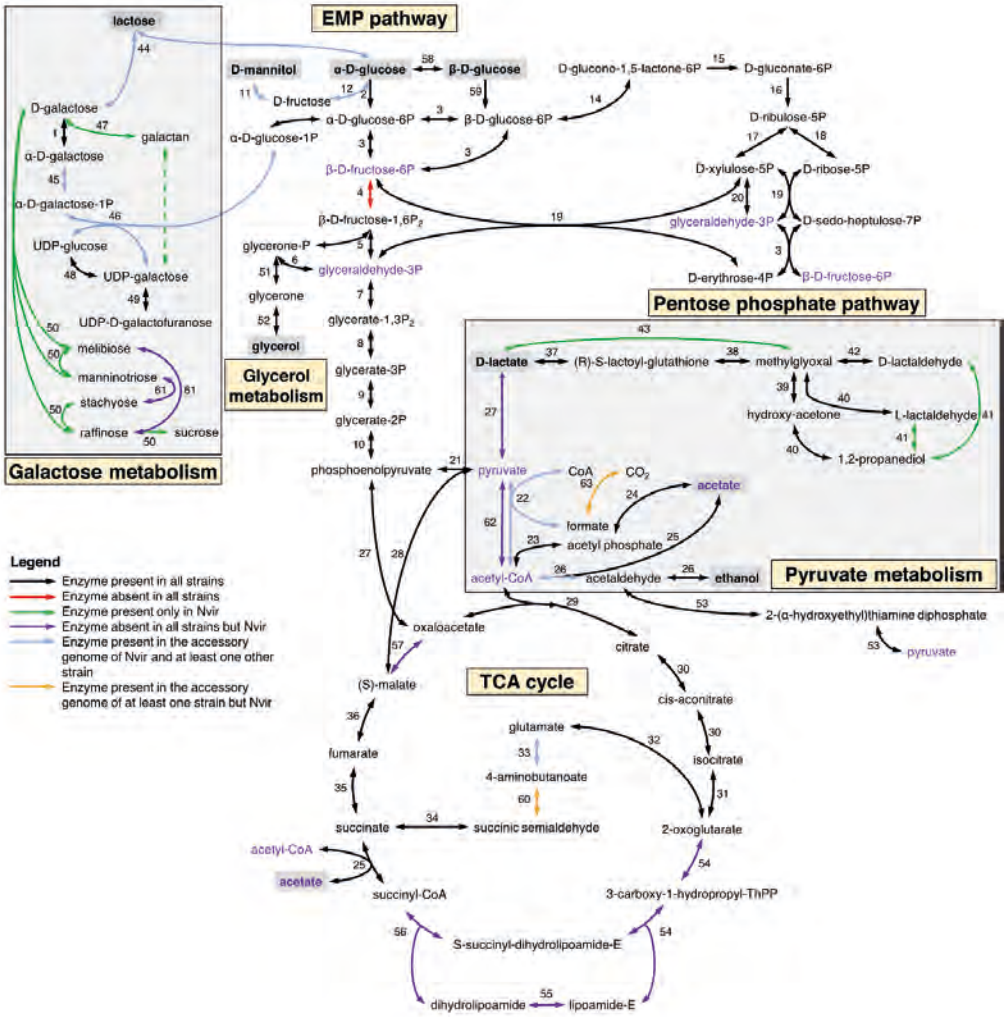

Figure 5.2. Comparative analysis of central carbon metabolism of *Commensalibacter* species. Characters in grey boxes indicate entry points for key metabolites that can be viewed in the KEGG mapper. Grey boxes indicate major metabolites and purple-coloured compounds that appear more than once in the diagram. **Black arrows** indicate enzymatic reactions of all strains; **red arrows** indicate the absence of an enzyme in all strains; **green arrows** indicate enzymatic reactions limited to strain Nvir; **blue arrows** indicate enzymatic reactions in the accessory genome of Nvir and/or AMU001, ESL0284, MX01, DmL 052 and A911^{T}; **purple arrows** indicate enzymatic reactions found in all strains except in Nvir; **orange arrows** indicate reactions found in the accessory genome of AMU001, ESL0284, MX01, DmL 052 and/or A911^{T}; non-solid arrow indicate the indirect link or unknown mechanism. Legend: (1) Aldose 1-epimerase; (2) glucokinase; (3) transaldolase / glucose-6-phosphate isomerase; (4) fructose-1,6-bisphosphatase II; (5) fructose-bisphosphate aldolase, class I; (6) triosephosphate isomerase; (7) glyceraldehyde 3-phosphate dehydrogenase; (8) phosphoglycerate kinase; (9) 2,3-bisphosphoglycerate-

independent phosphoglycerate mutase; (10) enolase; (11) D-arabinitol 4-dehydrogenase; (12) xylose isomerase; (13) phosphomannomutase / phosphoglucomutase; (14) glucose-6-phosphate dehydrogenase (15) 6-phosphogluconolactonase; (16) 6-phosphogluconate dehydrogenase; (17) ribulose-phosphate 3-epimerase; (18) ribose 5-phosphate isomerase A; (19) transketolase; (20) xylulose-5-phosphate/fructose-6-phosphate phosphoketolase; (21) pyruvate kinase; (22) formate C-acetyltransferase; (23) phosphate acetyltransferase; (24) acetate kinase; (25) succinyl-CoA:acetate CoA-transferase; (26) acetaldehyde dehydrogenase / alcohol dehydrogenase; (27) phosphoenolpyruvate carboxylase; (28) malate dehydrogenase (oxaloacetate-decarboxylating; NADP+); (29) citrate synthase; (30) aconitate hydratase; (31) isocitrate dehydrogenase (NAD+); (32) glutamate synthase complex (NADH) (33) glutamate decarboxylase; (34) succinate-semialdehyde dehydrogenase / glutarate-semialdehyde dehydrogenase; (35) succinate dehydrogenase / fumarate reductase, flavoprotein subunit, iron-sulfur subunit, cytochrome b subunit and membrane anchor subunit; (36) fumarate hydratase, class II; (37) hydroxyacylglutathione hydrolase; (38) lactoylglutathione lyase; (39) NADP-dependent alcohol dehydrogenase; (40) glycerol dehydrogenase; (41) lactaldehyde reductase; (42) glyoxylate/hydroxypyruvate/2-ketogluconate reductase; (43) D-lactate dehydratase / protein deglycase; (44) beta-galactosidase; (45) galactokinase; (46) UDP-glucose--hexose-1-phosphate uridylyltransferase; (47) UTP--glucose-1-phosphate uridylyltransferase; (48) UDP-glucose 4-epimerase; (49) UDP-galactopyranose mutase; (50) alpha-galactosidase; (51) triose/dihydroxyacetone kinase / FAD-AMP lyase (cyclizing); (51) glycerol dehydrogenase; (52) glycerol dehydrogenase; (53) pyruvate decarboxylase; (54) 2-oxoglutarate dehydrogenase E1 component; (55) dihydrolipoyl dehydrogenase; (56) 2-oxoglutarate dehydrogenase E2 component; (57) malate dehydrogenase; (58) aldose 1-epimerase; (59) glucokinase; (60) 4-aminobutyrate aminotransferase; (61) beta-fructofuranosidase; (62) pyruvate ferredoxin oxidoreductase; (63) formate dehydrogenase.

The TCA cycle is complete in all *Commensalibacter* strains except for strain Nvir, which lacks several enzymes. There are two alternative routes, which strain Nvir might take to complete the TCA cycle. The first half-cycle proceeds in a traditional way, from citrate to 2-oxoglutarate. Then, the 2-oxoglutarate dehydrogenase (OGDH) complex, lipoamide-E, and thiamine diphosphate (TPP) bind TPP to 2-oxoglutarate (Figure 5.2). The second half cycle starts from succinyl-CoA which is converted to succinate by the characteristic AAB enzyme, succinyl-CoA: acetate CoA-transferase, encoded by gene *aar*C. This, and the following step could be performed by all *Commensalibacter* strains leading to the formation of malate. The Nvir strain lacks membrane-bound malate dehydrogenase to convert malate to oxaloacetate but carries soluble malate dehydrogenase to convert malate to pyruvate generating one NADPH, thereby closing the TCA cycle.

Commensalibacter oxidizes ethanol under oxic and anoxic conditions

Like other AAB, *Commensalibacter* has the potential to perform oxidative fermentation of ethanol to acetaldehyde using promiscuous enzymes with

alcohol dehydrogenase (ADH) activity. All strains have a YiaY- and YqhD-like ADH encoded in their core genome, with YiaY possibly oxidizing ethanol and YqhD ADH favouring C4 and longer alcohols (Sulzenbacher et al., 2004). In addition, strains Nvir, MX01, A911^T, and DmL 052 harbour a bifunctional AdhE-like enzyme that has ADH and acetaldehyde dehydrogenase (ACDH) activity and is known to oxidize ethanol under aerobic conditions (Pineda et al., 2013). Strains MX01, A911^T, and DML 052 encode two additional ADHs of which one appeared to be the second subunit of the membrane-bound PPQ-ADH complex, which is a distinctive complex for AAB that links ethanol oxidation to the aerobic respiratory chain and consists of up to three subunits (Yakushi & Matsushita, 2010). The second subunit does not exhibit oxidoreductase activity but acts as an electron transporter within the complex. The first or third subunit is, however, missing in these strains, likely rendering the PPQ-ADH complex non-functional.

Commensalibacter can biosynthesize amino acids and scavenge B vitamins

As a member of the insect gut community, *Commensalibacter* has the potential to fulfil host nutritional requirements by supplementing amino acids and vitamins (Coolen et al., 2022). The core genome of *Commensalibacter* harbours all genes required for the biosynthesis of seven essential amino acids (valine, isoleucine, tryptophan, histidine, threonine, lysine, arginine) and eight non-essential amino acids (serine, cysteine, glycine, aspartate, asparagine, glutamate, glutamine, and tyrosine; Supplementary Table S3). *Commensalibacter* also had the genetic potential to synthesise aromatic amino acids, such as tryptophan, tyrosine, and phenylalanine via the shikimate pathway, which provides an alternative route to the formation of aromatic compounds (Rubin-Pitel et al., 2007). Moreover, all strains except Nvir harbour the genetic potential to biosynthesize proline, leucine, and methionine. Interestingly, *Commensalibacter* sp. Nvir may synthesize threonine differently than other strains since the canonical *thr*ABC operon was found absent in the MAG but a gene was found encoding for a threonine aldolase that enables its production from glycine and acetaldehyde. Along with the gene loss this suggests that *Commensalibacter* sp. Nvir is undergoing a reorganization of the genome, possibly as a result of its symbiotic lifestyle.

Considering B vitamins, *Commensalibacter* strains can potentially biosynthesize riboflavin, molybdopterin, and various porphyrins but have lost the ability to *de novo* biosynthesize thiamine, nicotinamide, pantothenate, biotin, and folate. They lost the ability to biosynthesize thiamine, nicotinamide, pantothenate,

biotin, and folate, possibly as a result of adaptation or to save energy but instead, they harbour scavenging systems to obtain these vitamins. Strains Nvir, AMU001, and ESL0284 have two additional genes that encode for L-aspartate oxidase and a quinolinate synthase, which enables *de novo* biosynthesis of NAD^+ from aspartate and glycerone-P. In a further step, NAD^+ is used to biosynthesize nicotinamide. Despite having additional genes, AMU001 and ESL0284 are likely not capable of nicotinate production, since they lack nicotinamidase that converts nicotinamide to nicotinate making Nvir the only strain potentially capable of *de novo* biosynthesis of NAD^+ from aspartate and glycerone-P.

Furthermore, riboflavin (vitamin B2) could be produced from either GTP or ribulose 5-phosphate, but the *Commensalibacter* strains are missing the phosphatase that generates 5-amino-6-(ribityl-amino)uracil and could therefore not produce riboflavin from GTP. Thus, riboflavin could only be biosynthesized via ribulose-5-phosphate. The AMU001 and ESL0284 strains have a gene encoding an NADH-dependent flavin reductase that allows riboflavin and its two derivatives flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) to be actively reduced to the $FMNH_2$/$FADH_2$. Lastly, strains AMU001, ESL0284, MX01, and A911^T could biosynthesize cobalamin (vitamin B12) via enzymes encoded by the *cob* operon.

The genome of *Commensalibacter* sp. Nvir shows adaptations to a symbiotic lifestyle

Commensalibacter sp. Nvir is a novel *Commensalibacter* strain and the first to be associated with a pest insect, the Southern green shield bug *Nezara viridula*. Considering the agricultural importance of *Commensalibacter* sp. Nvir, we undertook a detailed analysis of its genome to shed light on its role in *N. viridula.* In addition to the unique CDS in strain Nvir, it shares numerous genes with the other *Commensalibacter* strains (Figure 5.1D). We first investigated the accessory genes shared with other *Commensalibacter* species and then focused on the unique genes for strain Nvir.

A surprising discovery was made in the shared gene content between strain Nvir, A911^T and DmL 052. These strains encode a glutamate decarboxylase that converts aspartate to β-alanine and executes a key step in by-passing the TCA cycle via the γ-amino butyric acid (GABA) shunt. GABA-modulating bacteria participate in modulating host-microbe signalling and thus potentially benefiting the host (Feehily & Karatzas, 2013; Quillin et al., 2021; Siragusa et al., 2007). Furthermore, Nvir, AMU001, and ESL0284 share a *nad*ABC operon,

which encodes for proteins involved in the *de novo* synthesis of NAD^+/$NADP^+$ from aspartate (Begley et al., 2001). Further genomic analysis revealed that all strains encode a Major Facilitator Superfamily (MFS) NarK transporter and a NarGHI complex to import extracellular nitrate and nitrite. However, only the Nvir, MX01, A911^T, and DmL 052 genomes encode a NADH-dependent nitrite reductase NirBD complex allowing for the complete reduction of nitrate to ammonia during assimilatory or dissimilatory nitrite reduction.

In addition, the genomes of Nvir, MX01, A911^T, and DmL 052 encode proteins involved in the stress response to phosphate starvation, since they harbour an inorganic phosphate (P_i) ABC transporter encoded in the *pts*ABCS operon, regulated by the *pho*AB that responds to environmental P_i concentrations (Hsieh & Wanner, 2010). These systems contribute to bacterial growth in an environment-specific manner, therefore bacteria harbouring this system can grow in P_i-limited conditions. The phosphate starvation system might be an adaptation of insect-associated microbes to the nutrient-poor and sugar-rich plant sap, which generally has a low concentration of phosphorus compared to plant tissues (Wang et al., 2021).

On the other hand, bacterial survival is important in establishing a stable symbiotic relationship with the host, which could be achieved by toxin-antitoxin systems (TAs). TAs are activated upon environmental stresses like nutritional starvation, antibiotic exposure and oxidative stress, to inhibit cellular processes and reduce metabolism to arrest growth (Gerdes et al., 2005; Lewis, 2007). We found that Nvir encodes four TAs: HicAB, DinJ-YafQ, RelBE, and MazE-MazF. The RelBE and DinJ-YafQ TAs are also present in strains MX01, A911^T, and DmL 052, whereas no TAs were found in strains AMU001 and ESL0284. The higher number of TAs in the accessory genome of Nvir may reflect harsher environmental conditions within the host's gut and indicates that strain Nvir may be more resilient to environmental stresses as opposed to other *Commensalibacter* strains.

Considering the unique CDS clusters of *Commensalibacter* sp. Nvir, most of the genes have an undefined function. However, among the annotated proteins were two Cas9 and one Cas1 and Cas2 CDSs, as well as various transcriptional regulators. Strain Nvir also carries two genes encoding for secretory immunoglobulin A-binding proteins *esi*B. This protein was previously found in pathogenic *Escherichia coli* in humans where it helps to evade the host immune system and increases bacterial adherence to the gut epithelium (Pastorello et

al., 2013). Whether a similar mechanism is used to invade insect cells remains unknown but it could explain the prevalence of *Commensalibacter* among *N. viridula* (Coolen, Rogowska-van der Molen et al. *under review*).

Strain Nvir seemed to also have an expanded carbohydrate metabolism in comparison to other *Commensalibacter* species, potentially enabling it to metabolize galactan, lactose, D-tagatose, amylose, starch, glycogen, dextrin, and inositol. At least 25 unique genes in Nvir encode proteins involved in the metabolism of lipopolysaccharides with complex sugars like L-fucose, L-rhamnose, L-lyxose, L-xylulose, and arabinan. Seven of those 25 genes are specifically involved in amino sugar and nucleotide sugar metabolism and allow the degradation of nucleotide sugars like N-acetyl-D-galactosamine. The Nvir strain also harbours genes encoding D-xylose-, L-fucose-, and galactose-proton symporters and various MFS transporters that allow the transport of metabolites across the membrane. Taken together, the analysis showed that *Commensalibacter* strains may have adapted to the host-specific lifestyle by acquiring various genes associated with symbiosis.

Plant toxin detoxification potential in *Commensalibacter* sp. Nvir

As stated previously, strain Nvir resides in an agricultural-important pest insect that may benefit from symbiotic microorganisms in its gut, since they may aid in the degradation of toxic secondary plant metabolites (Rogowska-van der Molen et al., 2023; van den Bosch & Welte, 2017). Harbouring plant toxin-degrading gut symbionts enables insects to infest toxic plants without being exposed to the adverse effect of toxins (Coolen et al., 2022; van den Bosch & Welte, 2017). The toxins α-chaconine and α-solanine are widely distributed steroidal glycoalkaloids produced in the Solanaceae family which can disturb the development and metabolism of insects by causing malfunctions of the midgut (Chowanski et al., 2016; Paudel et al., 2017). The detoxification of these glycoalkaloids is based on the stepwise removal of monosaccharides ultimately yielding non-toxic solanidine (Hennessy et al., 2020). Comparative genomic analysis of the detoxification capabilities of *Commensalibacter* revealed the presence of a β-galactosidase in strains Nvir and MX01, as well as β-glucosidase in strains Nvir, MX01, A911^{T}, and DmL 052 (Table 5.2). However, only *Commensalibacter* sp. Nvir encoded α-rhamnosidase. This indicates that Nvir has the metabolic potential to fully degrade α-chaconine and α-solanine and facilitate detoxification in the insect's gut.

Table 5.2. Detoxification potential of *Commensalibacter* strains to degrade α-chaconine and α-solanine. Enzymes present in the genome are indicated with "+" and absent with "-".

Enzyme	Gene name	Nvir	AMU001	ESL0284	MX01	A911^T	DmL 052
α-rhamnosidase	*rha*A	+	-	-	-	-	-
β-galactosidase	*bga*B	+	-	-	-	-	-
	*lac*Z	+	-	-	+	-	-
β-glucosidase	*bgl*A	-	-	-	+	+	+
	*bgl*B	+	-	-	+	+	+

Conclusion

The comparative genomic analysis demonstrates that the genus *Commensalibacter* comprises four insect-associated species, all of which have traits of a symbiotic lifestyle. Their genomes are small and have a low GC content which suggests genome reduction (Nikoh et al., 2011). This study highlights differences between strains, emphasizing the distinct placement of *Commensalibacter* found in *N. viridula*. *Commensalibacter* sp. Nvir is the most divergent among the analysed strains and encodes 28 strain-specific protein clusters that are absent in the other *Commensalibacter* strains. The analysis revealed that most of these unique genes participate in carbohydrate, amino acid and B vitamin metabolism. *Commensalibacter* sp. Nvir harbours the genomic potential for metabolizing D-galactose, melibiose, manninotriose, and raffinose, as well as lipopolysaccharides containing L-fucose, L-rhamnose, L-lyxose, L-xylulose, and arabinan. Unlike other strains, Nvir's genetic potential suggests that it metabolizes lactose in a unique manner, with D-lactate dehydratase and lactaldehyde reductase. This indicates that *Commensalibacter* sp. Nvir adapted to the host's diet by acquiring dedicated carbohydrate-metabolizing genes. It was previously shown that diet is the driving force in the evolution of symbiosis and gene acquisition might reflect the close relationship between the *Commensalibacter* strain Nvir and *N. viridula* (Amato et al., 2019; Martino et al., 2018). *Commensalibacter* sp. Nvir also acquired the ability to survive in low phosphate environments such as plant sap and shows several adaptations to living within the insect gut system by *e.g.* harbouring genes related to the adherence to the gut epithelium. Moreover, as the only strain, it shows the genetic potential to detoxify α-chaconine and α-solanine. This study broadens our understanding of *Commensalibacter*'s function in the insect gut and illustrates that *Commensalibacter* not only potentially provides nutrients and digests food but also has the potential to degrade toxic secondary plant metabolites.

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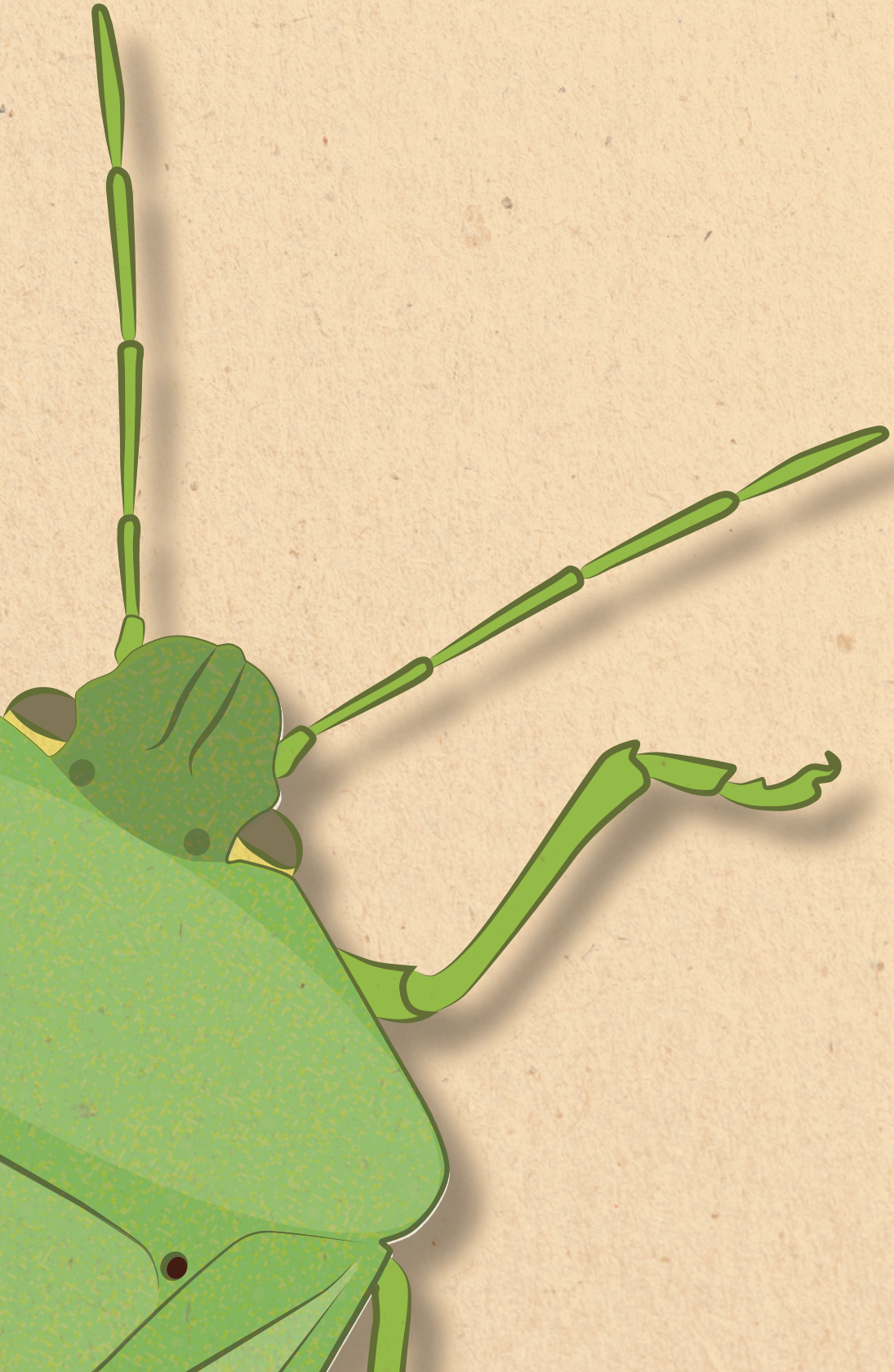

Chapter 6

Unveiling detoxifying symbiosis and dietary influence on the Southern green shield bug microbiota

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Abstract

The Southern green shield bug, *Nezara viridula*, is an invasive piercing and sucking pest insect that feeds on crops and poses a threat to global food production. Insects live in close relationships with microorganisms providing their host with unique capabilities, such as resistance to toxic plant metabolites. In this study, we investigated the resistance to and detoxification of the plant metabolite 3-nitropropionic acid by core and transient members of the *N. viridula* microbial community. Microbial community members showed a different tolerance to the toxin and we determined that six (out of eight) strains detoxified 3-nitropropionic acid. Additionally, we determined that 3-nitropropionic acid might interfere with the biosynthesis and transport of L-leucine. Moreover, our study explored the influence of diet on the gut microbial composition of *N. viridula*, demonstrating that switching to a single-plant diet retains yet shifts the abundance of core microbes. In line with this, testing pairwise microbial interactions revealed that core microbiota members support each other and repress the growth of transient microorganisms. With this work, we provide novel insights into the factors shaping the insect gut microbial communities and demonstrate that *N. viridula* harbours many toxin-degrading bacteria that could support its resistance to plant defences.

Introduction

The Southern green shield bug (*Nezara viridula*) is a piercing and sucking insect that feeds on plant sap of freshly developing seeds, plant tissues, and fruits, causing wilting and silver damage (Lucini & Panizzi, 2018; Medina et al., 2018; Todd, 1989). This pest insect is a global threat to diverse crops from the Fabaceae, Solanaceae and Brassicaceae families, which comprise economically and nutritionally important crops like soybean, tomato, or cabbage. With the increasing global demand for food production, decreasing crop losses has become an urgent challenge. Despite many efforts to decrease the number of pest insects like *N. viridula,* traditional pest control strategies with chemical insecticides have had a limited effect. Moreover, these chemical insecticides have a pronounced effect on human health and contribute to environmental pollution (Botías et al., 2019; Costa et al., 2007). Therefore, an effective and sustainable solution is needed to secure food production in the future. Recent studies demonstrated that targeting symbiotic microorganisms could be an alternative solution to decrease crop losses since gut microbiota supports insect resistance against toxic compounds and insecticides (Chung et al., 2018; Itoh et al., 2018; Medina et al., 2018; Rogowska-van der Molen et al., 2023; van den Bosch & Welte, 2017). While this is a promising approach to the development of sustainable pest management strategies, fundamental knowledge of the tri-trophic interactions between pest insects, their microbiota and host plants is lacking.

The insect microbiome consists of core and transient microbiota. Core microbiota are obligate and facultative symbionts that are relatively stable and regularly associated with the host, while the transient microbiota represents microbes that inhabit the host only for a rather short period. Previous studies showed that shield bugs acquire core symbionts via vertical transmission in the egg surface smearing process during oviposition. Transient members are, on the other hand, acquired from the environment or upon feeding (Frago et al., 2012; Oliver et al., 2010; Tada et al., 2011). Obligate symbionts represent the majority of the microbiota and are required for the survival and development of their host by supplementing amino acids and vitamins, while facultative symbionts constitute a minor part of the community and are not essential for host viability. Facultative symbionts can nevertheless support insect resistance to heat stress or pathogens (Capuzzo et al., 2005; Medina et al., 2018; Montllor et al., 2002; Ryu et al., 2008; Tada et al., 2011). Although transient microbes only constitute a small proportion of the microbiome (<1%), a recent study on

mosquitos showed that both core symbionts and transient microbes strongly affect insect reproductive cycles via the provision of B vitamins (Serrato-Salas & Gendrin, 2023). Therefore, transient microbes could be potentially as important as core symbionts. Understanding the interplay between core and transient microbiota members is thus crucial for comprehending the function of the insect microbial community.

Several components determine insect microbiota composition, such as diet, host phylogeny and microbe-microbe interactions (Amato et al., 2019; Colman et al., 2012). To sustain growth, microbes that live in consortia, such as those in the insect gut, might depend on each other by biosynthesizing and exchanging certain metabolites (Zhang et al., 2018). Besides this, the gut bacterial community composition of evolutionarily distinct insects is similar during feeding on the same diet, for example, Huang et al. (2021) determined that gut microbes facilitate convergent evolution during the adaptation to the same diet in phylogenetically distinct species. As a result, microbes seem to not only have an impact on insect viability and development but also adaptations to changing environments (Itoh et al., 2018; Sato et al., 2021; van den Bosch & Welte, 2017). One such adaptation is the evasion of plant defences by detoxification of phytochemicals (Bruce, 2015). A recent study indicated that potato tuber moth microbiota mediated detoxification of α-chaconine and α-solanine by harbouring gene clusters involving α-rhamnosidase, β-glucosidase and β-galactosidase (W. Wang et al., 2022) and cabbage root fly larvae microbiota contributed to 2-phenylethyl isothiocyanate degradation by harbouring isothiocyanate hydrolase enzymes in plasmids (Welte, de Graaf, et al., 2016; Welte, Rosengarten, et al., 2016).

The core microbiota of *N. viridula* has previously been shown to degrade isoflavonoids and deactivate soybean protease inhibitors, indicating that they might facilitate *N. viridula*'s resistance to plant chemical defences (Medina et al., 2018). However, the role of transient microbiota remains elusive. Besides isoflavonoids, leguminous plants biosynthesize other defence compounds, such as 3-nitropropionic acid (NPA), to deter insects. When ingested by mammals, NPA causes irreversible inhibition of succinate dehydrogenase in the tricarboxylic acid cycle (TCA), impairing the function of the electron transport chain and causing the accumulation of toxic nitrite ions that disrupt haemoglobin (Anderson et al., 2016; Francis et al., 2013). Several soil- and gut-associated bacteria were reported to degrade NPA using either propionate-3-nitronate monooxygenase (P3N), nitronate monooxygenase (NMO) or putative nitronate

monooxygenase (PNMR), encoded by *pno*A, *nmo*A or *pnm*R, respectively (Anderson et al., 2000; Nishino et al., 2010; Rogowska-van der Molen et al., 2022). A recent study demonstrated that *N. viridula* that feeds on the NPA-rich legume vine crown vetch (*Securigera varia*) showed no symptoms of intoxication and that the isolated gut microbe *Pseudomonas* sp. Nvir was able to detoxify NPA with the release of CO_2, nitrite and nitrate (Rogowska-van der Molen et al., 2022). Given the apparent resistance of *N. viridula* to NPA, we studied the possible involvement of core and transient gut-associated microbiota in the degradation of NPA.

To investigate the mechanistic role of the *N. viridula* gut microbiota on the toxin resistance of its host, we investigated previously identified core members and newly isolated transient gut microbiota by first evaluating the presence of detoxifying genes in genomes and plasmids with genome sequencing. Additionally, we cultivated eight gut isolates to assess their resistance and ability to metabolize NPA. These analyses indicated that the gut isolates had a different resistance pattern to NPA even though most were capable of NPA detoxification and encoded either *pno*A, *nmo*A, or *pnm*R in their genomes. Transcriptomic and metabolomic analyses of the core microbe *Pantoea* suggested a possible negative influence of NPA on amino acid metabolism and transport. In addition, to assess whether diet plays a role in altering bacterial community, insects were fed for six months with either black mustard (Brassicaceae) or black nightshade (Solanaceae) plants and afterwards, their gut microbiota was compared to insects feeding from multiple plants. Our findings indicated that switching to a single-plant-based diet caused a shift in the gut core microbiota of *N. viridula*. Interestingly, experiments testing pairwise interactions between the isolated microbial community members showed that the core microbiota had many mutualistic microbe-microbe interactions and suppressed the growth of transient bacteria. Together, we provide insights into the influence of dietary components on the insect gut microbial community composition and showed that *N. viridula* harbours many toxin-degrading bacteria that could support its resistance to plant defence metabolites.

Materials and Methods

Insect collection and rearing

Nezara viridula were collected in the field from creeping thistle (*Cirsium arvense*) in the Netherlands (51.348028, 6.128802) and were provided by

Wageningen Plant Research (Bleiswijk, the Netherlands) in March 2023. The insects were transferred to a greenhouse and placed in a rearing cage to establish a colony (native population). *N. viridula* individuals were reared in a greenhouse facility with normal daylight and additional light to obtain a photoperiod of 16:8h (light:dark) year-round. Insects were provided with sunflower (*Helianthus annuus*), soybean (*Glycine max*), brown mustard (*Brassica juncea*) seeds, flat beans (*Phaseolus vulgaris*), and the native plants crown vetch (*Securigera varia*), black mustard (*Brassica nigra*) and black nightshade (*Solanum nigrum*).

Insect dissection and pure culture isolation

Five complete gut systems (M1-M4 sections) and salivary glands of adult *N. viridula* were dissected directly after submersion of insects in 70% ethanol for approximately one min after which movement stopped. Dissection was performed under non-sterile conditions using a stereomicroscope, scalpel, and forceps. Separation of tissues from the insect body was performed in phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4) to prevent rupture of the delicate tissue.

For culturing and isolation, salivary glands and complete gut systems were disrupted in 200 µL PBS by vortexing. Saliva and frass samples were collected from adult *N. viridula* as described previously (Coolen, Rogowska-van der Molen et al., *under review*). Next, samples were diluted and plated on either Luria-Bertani (LB) agar (0.5% peptone, 0.3% yeast extract, 0.5% NaCl and 1.5% agar) or mannitol agar (2.5% *n*-mannitol, 0.5% yeast extract, 0.3% peptone and 1.5% agar) plates or added to M9 mineral salt medium (33.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.55 mM NaCl, 9.35 mM NH_4Cl, 1 mM $MgSO_4$, 0.3 mM $CaCl_2$, Thauer vitamin mixture (Sowers & Ferry, 1985), trace elements (Kurth et al., 2019), pH 7.2) containing either 100 µM of α-solanine or 3-nitropropionic acid (NPA) as a carbon source. Agar plates were incubated at 30°C for a maximum of three weeks after which single colonies were transferred to new LB agar plates at least six times before they were considered pure. Liquid cultures were incubated at 30°C at 200 rpm for two weeks and afterwards diluted and plated on M9 mineral salt medium agar plates containing either 100 µM of α-solanine or 3-nitropropionic acid and incubated at 30°C for one week. Colonies were transferred to new M9 agar plates at least six times before they were considered pure.

Dietary influence on microbiota composition

Fifteen *N. viridula* (1st-5th instars and adults) individuals were collected in May 2023 from the native population and placed in separate rearing cages that contained either black mustard or black nightshade plants. Insects were reared for six months and afterwards, three *N. viridula* adults were collected from black mustard, black nightshade, and native population (control), and dissected. DNA was extracted from complete gut systems (M1-M4 sections and hindgut) for determination of gut microbial profile with 16S rRNA gene amplicon sequencing.

As a control, to determine the plant microbiome, non-infested black mustard and black nightshade plants (five grams each) were harvested and washed for 30 min in phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4) with six one-minute vortex periods. Tubes were spun down for 15 min at 3200 rpm and pellets were used for DNA isolation.

DNA isolation, genome sequencing and data analysis

DNA was isolated from all pure cultures, dissected gut and plant, using a DNeasy PowerSoil kit (QIAGEN, the Netherlands) according to the manufacturer's protocol. For pure cultures, 1 mL of pelleted cells were lysed with the TissueLyser LT (QIAGEN) for 2 min at 50 Hz. MiSeq sequencing (Illumina, San Diego, CA, USA) was performed with the Nextera XT Library Preparation Kit (Illumina, San Diego, California, United States) according to the manufacturer's instructions. Paired-end sequencing (2x 300bp) was performed using the Illumina MiSeq sequencer (Illumina) and the MiSeq Reagent Kit v3 (Illumina) according to the manufacturer's protocol. Long-read sequencing was performed with the MinION R9 flow cell (FLO-MIN106; Nanopore, Oxford, UK), according to the manufacturer's protocol, using unsheared DNA (no size selection) and NEBNext formalin-fixed, paraffin-embedded (FFPE) repair mix (M6630), a ligation sequencing kit (SQK-LSK109), the NEBNext end repair/dA-tailing module (E7546), a flow cell priming kit (EXP-FLP001), NEB Blunt/TA ligase master mix (M0367), the NEBNext quick ligation module E6056, and barcode kit EXPNBD104. The libraries were checked for quality and size distribution using the Agilent 2100 Bioanalyzer and the High sensitivity DNA kit (Agilent Technologies, Santa Clara, California, USA). Quantitation of the libraries was performed by the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc Waltham USA).

Oxford Nanopore reads were trimmed to remove adapters using Porechop (0.2.3_seqan2.1.1) (Wick et al., 2017a) and assembled with Flye (2.9) (Kolmogorov et al., 2019). Sequences were aligned with Minimap2 (2.22) (Li, 2018) and consensus assemblies were generated with Racon (v1.4.20) (Vaser et al., 2017). Quality trimming and filtering of MiSeq Illumina reads was performed with BBDuk (BBTools v38.75) (Bushnell, 2014) and extracted reads were aligned with BBMap (v38.75). Genomes were assembled using MiSeq Illumina and Nanopore reads with Unicycler (v.0.4.4) (Wick et al., 2017b). The taxonomy and the quality of the generated genomes were assessed through a single-copy marker gene analysis CheckM (1.1.2) (Parks et al., 2015). Genomes were annotated with Prokka (1.13.4) (Seemann, 2014). Metabolic pathways were predicted with KEGG (Kanehisa & Goto, 2000).

16S rRNA gene amplicon sequencing and analysis

The gut bacterial community of *N. viridula* was determined by amplification of the V3-V4 region of the 16S rRNA gene. The sequencing was performed by Macrogen (the Netherlands) with *Bac341F* and *Bac806R* primers (Caporaso et al., 2011; Herlemann et al., 2011) using an Illumina MiSeq sequencer (Illumina). Paired-end (2x 301bp) reads libraries were prepared with the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina).

The quality of the raw paired-end sequences was checked with FastQC 0.11.8 (Bushnell, 2014). The reads were then filtered, and adapters were trimmed. Approximately 40,000-70,000 paired-end sequencing reads were obtained per sample. The data were further processed using the DADA2 1.8 pipeline (Callahan et al., 2016) in R. Phylogenetic taxonomy of the reads was assigned using the SILVA 16S rRNA database 138.1 (Quast et al., 2012). Count data were normalized to relative abundance. Microbial community profiles were analysed and amplicon sequence variants (ASVs) were visualized using the phyloseq (McMurdie & Holmes, 2013) and ggplot2 (Wickham, 2009) packages in R.

RNA isolation and transcriptome sequencing

Pantoea sp. Nvir was cultured in 150 mL M9 mineral salt medium supplemented with glucose 0.08% for 24 hours. Upon glucose depletion, either 100 µM of NPA (treated) or glycerol (control) was supplemented to the culture. Biomass samples for RNA extraction were taken 2h after the supplementation. Samples (5 mL) were centrifuged at 3500 × *g* for 10 min at 4°C and RNA was extracted with the RiboPure™-Bacteria Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Prior to sequencing, bacterial mRNA was purified

with the MICROBExpress™ Bacterial mRNA Enrichment Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Transcriptome libraries were constructed using the TruSeq® Stranded mRNA Library Prep protocol (Illumina) according to the manufacturer's instructions. Obtained libraries were checked with Qubit as described before. Equimolar pooled libraries were sequenced using the Illumina MiSeq sequencer (Illumina). For sequencing, the 150 bp sequence chemistry was performed using the MiSeq Reagent Kit v3 (Illumina) according to the manufacturer's protocol in one direction. For both control and treated cultures, RNA was extracted from three independent biological replicates.

The quality of Illumina single-end transcriptome raw reads was assessed with FASTQC (0.11.8) (Bushnell, 2014) prior to reads mapping. Single-end reads were mapped against the annotated genome using Kallisto (0.46.2) (Bray et al., 2016) and outputs were summarized with MultiQC 1.11 (Ewels et al., 2016). Read counts were transformed in Tximport (Soneson et al., 2015) for differential gene expression analysis using BioConductor packages in R environment (Huber et al., 2015). To determine differentially expressed genes log2-fold change cut-off of 2 was set. In addition, to address the issue of multiple testing, a false discovery rate (FDR) correction was performed with *a* $p < 0.05$ value using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995).

3-nitropropionic degradation assay

The axenic cultures of bacterial isolates were pre-grown aerobically in batch (50 mL; $n = 3$) for 24 hours (30°C, 200 rpm) in M9 mineral salt medium as described earlier, until OD_{600} of 0.7 ± 0.1. Hereafter, 100 µM of NPA was supplemented to the pre-grown culture. For the determination of NPA, nitrite (NO_2^-), and nitrate (NO_3) concentrations, 1 mL samples were collected for 24 hours. The supernatant was collected after centrifugation at 20,000 × *g* for 3 min and was stored at -20°C until analysis.

High-performance liquid chromatography (HPLC) was used to determine 3-nitropropionic acid concentrations. Using an Agilent 1100 Series LC system equipped with a diode array detector and a C18 column (LiChrospher 100 RP-18 end-capped (5µm) column, 125 mm×4 mm, Merck), gradient analysis was performed with a flow rate of 1.2 mL min^{-1} using 0.1% orthophosphoric acid in water (Solvent A) and acetonitrile (Solvent B). The gradient analysis was performed with 0% Solvent B at 0 to 5 min, 10% Solvent B at 5 to 6 min, 45% Solvent B at 6 to 8 min, 85% Solvent B at 8 to 10 min, 90% Solvent B at 10 to 12

min, 90% Solvent B at 12 to 13 min, 0% Solvent B at 13 to 15 min. Before the analysis, 200 µL of supernatant was acidified with 25 µL 1 M sulphuric acid, after which 100 µL of the sample was injected. NPA was measured at 210 nm with a retention time of 2.9 min.

To determine nitrite (NO_2^-) and nitrate (NO_3^-) concentrations, a Griess-reagent assay was used with standard curves of $NaNO_3$ and $NaNO_2$. The supernatant (100 µL) collected during the NPA degradation assay was transferred to a 96-well plate containing 100 µL of Griess reagent (50 µL of reagent A: 1% (w/v) sulfanilic acid in 1 M HCl and 50 µL of reagent B: 0.1% (w/v) naphthyl ethylene diamine dihydrochloride in demineralized water). The plate was incubated for 10 min at room temperature and absorbance was measured at 540 nm with a microplate reader to determine nitrite (SpectraMax 190, Molecular Devices, San Jose, California, United States). Next, 27 µL of VCl_3 (10 mg mL^{-1} in 1 M HCl) was added to the sample to reduce nitrate to nitrite. Subsequently, the 96-well plate was incubated in the dark at 60°C for 30 min, whereafter absorbance was measured at 540 nm.

LC-qToF-MS untargeted metabolomics

To determine the metabolome of *Pantoea* sp. Nvir under NPA-supplemented conditions, metabolites were extracted from a 5 mL sample (n = 4; treated and control samples) obtained after 6 hours of NPA supplementation as described under the NPA degradation assay section. Cell pellets were weighed and equivalent amounts of approximately 500 µL acetonitrile:methanol:H_2O (40:40:20; v:v:v) were added to each sample and vortexed vigorously for 10 s. Samples were incubated for 5 min on ice and subsequently centrifuged at 20 000 x *g* for 5 min. The supernatant was transferred into a new tube and stored at -70°C until metabolite analysis.

Samples were analysed with a 1290 Infinity II LC system coupled to a 6546 Q-ToF MS (Agilent Technologies) (LC/Q-ToF-MS) as described (Jansen et al., 2020). In short, a 2 µL sample was injected onto a Diamond Hydride Type C column (Cogent) and separated using a 0.4 mL min^{-1} gradient of water with 0.2% formic acid (A) and acetonitrile with 0.2% formic acid (B). The gradient was as follows: 0–2 min: 85% B, 3–5 min: 80% B, 6–7 min: 75% B, 8-9 min: 70% B, 10–11 min: 50% B, 11–14: 20% B, 14–24: 5% B, followed by 10 min re-equilibration at 85% B. Detection of compounds was performed from m/z 50-1200 in the positive and negative ionization modes. Raw data were converted to mzXML format using ProteoWizard software (Chambers et al., 2012) and two

sample groups were compared using XCMS online with centWave method and set parameters (15 ppm, 10-120 peak width, Signal/Noise threshold 6), and obiwarp retention time correction (profStep 1). The peak table was filtered for features that were more abundant in the NPA-treated samples compared to controls ($p < 0.01$, max intensity $>10^6$). From the resulting list, four features from positive and ten from negative ionization modes, respectively, were further analysed. For identification, MS/MS fragmentation spectra were obtained using the LC-qToF-MS method described above, operated at a collision energy of 10, 20 and 40 V. For metabolite annotation, fragmentation spectra were queried in the MassBank and MoNA databases (August 2023) (Horai et al., 2010; *MoNA - MassBank of North America*, 2007). To validate the putative identification of 2-isopropylmalate, a chemical standard was obtained (>98%; Sigma Aldrich, the Netherlands), followed by a comparison of the fragmentation spectra between *Pantoea* sp. Nvir metabolite and standard compound. A mirror plot was generated with a Spectra package in R with a match tolerance of 0.1 ppm.

To identify the NPA influence on the L-leucine biosynthesis pathway, the relative abundance of 3-hydroxy-3-methyl-2-oxobutanoate, 2-oxoisovalerate, 2-isopropylmalate (putatively annotated based on accurate mass) and L-leucine was determined using Qualitative Analysis 10.0 (Agilent Technologies).

Determination of resistance against 3-nitropropionic acid

Resistance of microbial isolates against NPA was evaluated with disc diffusion assays. Bacterial cultures were pre-grown in LB medium (30°C, 200 rpm) for 24 hours or 168 hours (for *Sodalis* sp. Nvir) and subsequently diluted to OD_{600} of 0.1 ± 0.05. An aliquot of 100 µL of the culture was plated on LB agar plates ($n = 2$). A paper disc (6 mm diameter, Whatman) soaked with 10 µL of NPA solution (0, 93.75, 187.5, 375, 750 and 1000 mM, dissolved in dimethylsulfoxide) was placed on the plate. Plates were incubated for 48 hours at 30°C and afterwards, the diameter of inhibition was measured.

Pairwise species interactions on phloem-sap medium plates

The growth of bacterial pure cultures in phloem-sap (PS) medium (100 mM L-serine, 100 mM methionine and 38.5 mM aspartic acid (pH=7.0, adjusted with NaOH)) (Tan et al., 2016) was determined in varying sucrose concentrations (0.5, 1, 2, 5, 10, 20% (v/v) sucrose) by measuring the OD_{600} for 114 hours in a microplate reader (SpectraMax 190, Molecular Devices). Cultures were pre-grown for 24 hours or 168 hours (for *Sodalis* sp. Nvir) in LB medium (30°C, 200 rpm), and accordingly diluted to an OD_{600} of 0.05 and inoculated in a 96-

well plate to a well containing 200 µL PS medium (n = 3). Optical density was measured in 15-minute intervals. According to the growth curve, PS medium with 10% sucrose solution was favoured by all bacterial isolates and therefore this sucrose concentration was used further on to determine pairwise species interactions on PS agar plates.

The pairwise (binary) microbial interactions were assessed in 10% sucrose PS agar plates (1.5% agar) containing 0.03 g L^{-1} bromocresol green as the stain. Per background species, an equivalent volume needed for OD_{600} = 5 was collected by centrifugation at 16 000 x g at 3 min. Cell pellets were resuspended in 150 µL of 0.9% NaCl and spread on PS agar using sterile glass beads (n = 2). Plates were incubated for 2 hours at 30°C before pinning to provide growth advance to the background species. Query (pinned) species were prepared by centrifugation (16 000 x g, 3 min) of 2 mL cultures and pellets were resuspended in 300 µL 0.9% NaCl. After a 2 hours incubation period, using a multichannel pipette, 5 µL of the query species was pinned on top of the background species (n = 4). In addition, plates with no background species were prepared and pinned as described earlier (n = 4). The plates were incubated for 48 hours at 30°C and the plates were imaged using Samsung Galaxy S22 Ultra 108-megapixel camera. The colony surface area was determined using ImageJ (version 2.35). The growth effects were classified as positive, negative, or neutral if the colony surface area was significantly (two-sided t-test, p-value cut-off = 0.05) larger, smaller, or similar in comparison with plates without background species. Based on this, the type of interaction between two species was given, based on the reciprocal effects, namely mutualism, commensalism, amensalism, parasitism and competition.

Results

Genomic and functional characterization of *Nezara viridula* microbiota reveals nutrient production and detoxification abilities

Nezara viridula core microbiota consists of four bacterial genera, namely *Pantoea, Sodalis, Serratia* and *Commensalibacter*, which represent >99% of the gut microbial community (Coolen, Rogowska-van der Molen et al., *under review*). The remaining fraction represents a low abundant transient microbial community. In this study, we sequenced previously obtained pure cultures (Coolen, Rogowska-van der Molen et al., *under review*) and new species isolated from the gut, frass, and saliva of *N. viridula*. The genomic characteristics of core and transient *N. viridula* microbiota are summarized in Table 6.1. Both core

(*Pantoea* sp. Nvir, *Serratia marcescens* (S-F1) and (S-F5), *Sodalis* sp. Nvir) and transient (*Bacillus megaterium* (S-ITC1), *Bacillus frigoritolerans* (S-Sol1), *Klebsiella pneumoniae*, *Pseudomonas* sp. Nvir) microbiota members showed differences in genome size, number of plasmids and GC content. Genomes of bacteria belonging to the core microbiota were large with a GC content varying between 55-60%, and harboured multiple plasmids. Of all bacteria belonging to the core microbiota, *Pantoea* sp. Nvir had the smallest genome, but the largest plasmid with 508,307 bp. Interestingly, plasmids A and B of *Pantoea* had many genes encoding carbohydrate-active and plant metabolite detoxification enzymes (quercetin 2,3-dioxygenase, phenolic acid decarboxylase and putative tartrate decarboxylase). Both *Serratia* marcescens strains had similar genome sizes and numbers of coding sequences (CDS). The analysis also showed that *Sodalis* sp. Nvir, which is known to colonise salivary glands nearly entirely, had a large genome that included genes for flagellar assembly indicating the ability to adapt to a free-living lifestyle. On the other hand, representatives of the transient microbiota had large genomes and considerably divergent GC content ranging from 37-62%. As opposed to *B. megaterium* and *Pseudomonas*, only *B. frigoritolerans* and *K. pneumoniae* had four and one plasmids, respectively. Overall, the genomes and plasmids of core and transient microbes seem to be highly similar in terms of size and number of CDS and further functional analysis of symbiosis-associated genes could unveil their role in *N. viridula* gut.

After determining the basic genomic characteristics, we investigated the microbial nutrient production potential and found that all bacteria associated with the core microbiota as well as *Klebsiella* from the transient microbiota had the genomic potential to biosynthesize all essential amino acids, namely valine, leucine, isoleucine, threonine, methionine, arginine, lysine, histidine, phenylalanine, and tryptophan. Bacteria of both core and transient microbiota could additionally synthesize vitamins B1, B2, B5, B6 and B7, based on their genomic potential (Supplementary Table S1). Since many insects benefit from symbiotic microorganisms to detoxify phytotoxins and pesticides (Itoh et al., 2018; van den Bosch & Welte, 2017), we inspected the potential of *N. viridula* microbiota to degrade toxins present in the diet of *N. viridula.* In our rearing facility, insects feed on black mustard, black nightshade and crown vetch which synthesise isothiocyanates (ITC), solanaceous glycoalkaloids, and 3-nitropropionic acid (NPA), respectively to deter insects (Al-Snafi, 2016; Fahey et al., 2001; Mohy-ud-Din et al., 2010). No member of the microbiota harboured the *sax*A gene encoding isothiocyanate hydrolase responsible for the degradation of 2-phenylethyl isothiocyanate (Welte, Rosengarten, et al., 2016), however several

Table 6.1. Summary of *Nezara viridula* core and transient microbiota genomic characteristics

	Species	Type of DNA	Size (bp)	Number of segments	Status
Core microbiota	*Pantoea* sp. Nvir	Genome	3,954,582	1	Circular
		Plasmid A	508,307	1	Circular
		Plasmid B	200,700	1	Circular
		Plasmid C	50,023	1	Circular
	Serratia marcescens (S-F1)	Genome	5,201,660	1	Circular
		Plasmid A	148,677	1	Circular
		Plasmid B	105,264	1	Circular
		Plasmid C	100,281	1	Circular
	Serratia marcescens (S-F5)	Genome	5,201,368	1	Circular
		Plasmid A	105,712	1	Circular
		Plasmid B	105,264	1	Circular
	Sodalis sp. Nvir	Genome	5,497,650	44	Contigs
Transient microbiota	*Bacillus megaterium* (S-ITC1)	Genome	6,115,434	235	Contigs
	Bacillus frigoritolerans (S-Sol1)	Genome	5,646,898	1	Circular
		Plasmid A	248,967	1	Circular
		Plasmid B	143,153	1	Circular
		Plasmid C	96,428	1	Circular
		Plasmid D	16,027	1	Circular
	Klebsiella pneumoniae	Genome	5,400,374	1	Circular
		Plasmid A	281,938	1	Circular
	Pseudomonas sp. Nvir	Genome	5,856,348	1	Circular

microbes harboured genes involved in the partial degradation of α-chaconine and α-solanine, namely β-glucosidase (*bgl*B) and β-galactosidase (*lac*Z, *ebg*A, *bga*A, *cbg*A; Supplementary Table S2) (Hennessy et al., 2020). To metabolize NPA, either *pno*A, *nmo*A or *pnm*R encoding for propionate-3-nitronate monooxygenase and nitronate monooxygenases, respectively, must be present (Rogowska-van der Molen et al., 2022). Seven members of the *N. viridula* microbiota possessed at least one of those genes. *Sodalis* sp. Nvir seemed to be the only one lacking the inferred genomic potential to detoxify NPA.

Due to the widespread occurrence of NPA-degrading genes among the *N. viridula* microbiota, we analysed genes' phylogeny by performing a multiple sequence alignment of *pno*A, *nmo*A and *pnm*R amino acid sequences (Supplementary

GC (%)	Completeness (%)	Contamination (%)	CDS	rRNA	tRNA
59.0	99.99	0.66	3507	22	82
57.3			462		
56.1			183		
51.8			51		
59.9	99.8	0	4783	22	93
52.6			162		
53.6			110		
51.1			108		
59.9	99.8	0	4783	22	91
52.8			102		
53.6			110		
55.5	97.62	0	7519	21	67
37.3	99.43	1.87	6175	42	140
40.6	100	0	5410	39	77
36.8			259		
37.7			133		
36.4			108		
36.5			15		
57.6	100	0.43	4947	25	87
52.8			280		
62.1	99.61	3.92	5414	22	78

Figure S1B). The enzymes encoded by *nmo*A and *pno*A showed substantial similarity with a tendency to cluster together, and PnmR was markedly different. Also, genus-dependent clustering of NmoA and PnoA sequences was noticed in *Serratia* and *Bacillus*. *Pseudomonas* sp. Nvir harboured both *pno*A and *pnm*R but only the latter was transcribed during culturing with NPA (Rogowska-van der Molen et al., 2022). This underlines that the presence of a gene alone is not sufficient to demonstrate biodegradation activity. As most bacteria from both core and transient microbiota contained genes potentially enabling them to degrade NPA, we proceeded with their functional characterization of NPA resistance and degradation by the eight microbial isolates.

N. viridula microbiota detoxifies NPA *in vitro* and has variable NPA tolerance

The isolated microbial community members of the *N. viridula* gut microbiota were assessed for resistance against NPA with disc diffusion assays (Figure 6.1A; Supplementary Figure S2 and Table S3). *Klebsiella* and the two *Serratia* strains were most resistant and showed almost no inhibition (< 8 mm Ø zone of inhibition around the 6 mm Ø paper disc) even at the highest NPA concentration. *Pantoea*, *Pseudomonas*, and two *Bacillus* strains were less resistant to NPA and showed a diameter of inhibition varying from 9-12 mm. The most susceptible strain was *Sodalis,* whose diameter of inhibition at 1000 mM NPA reached 27 mm. Combining these results with the genomic analysis revealed that strains which harboured genes encoding for NPA-degrading enzymes were substantially more resistant to NPA than *Sodalis* which did not carry them.

To test whether the observed NPA resistance could indeed be caused by degradation, we next investigated whether members of the microbiota obtained from *N. viridula* detoxify NPA in cultures. All isolates were cultured in liquid M9 minimal salt medium supplemented with 100 µM NPA (Figure 6.2B). *Pantoea*, *Pseudomonas* and *B. frigoritolerans* demonstrated rapid NPA degradation within 24 hours, whereas both *Serratia* strains *and B. megaterium* only partially degraded NPA, leaving more than half of the NPA in the medium after 24 hours. All NPA-degrading strains exhibited prompt degradation in the beginning, followed by substantially reduced degradation after 8-10h. These observations might be the result of the accumulation of inhibitory by-products like nitrite and nitrate in the culture medium (Supplementary Figure S3A-B, S5). While our genomic analysis indicated that *Klebsiella* harboured *pno*A and therefore has the potential to metabolize NPA, NPA degradation was not observed under the culturing conditions chosen in this study. *Sodalis* did not exhibit the capability to degrade NPA either, as expected from the genomic predictions. Altogether, our genomic analysis largely aligned with the observed biodegradation pattern and showed that both core and transient microbiota members were able to detoxify NPA.

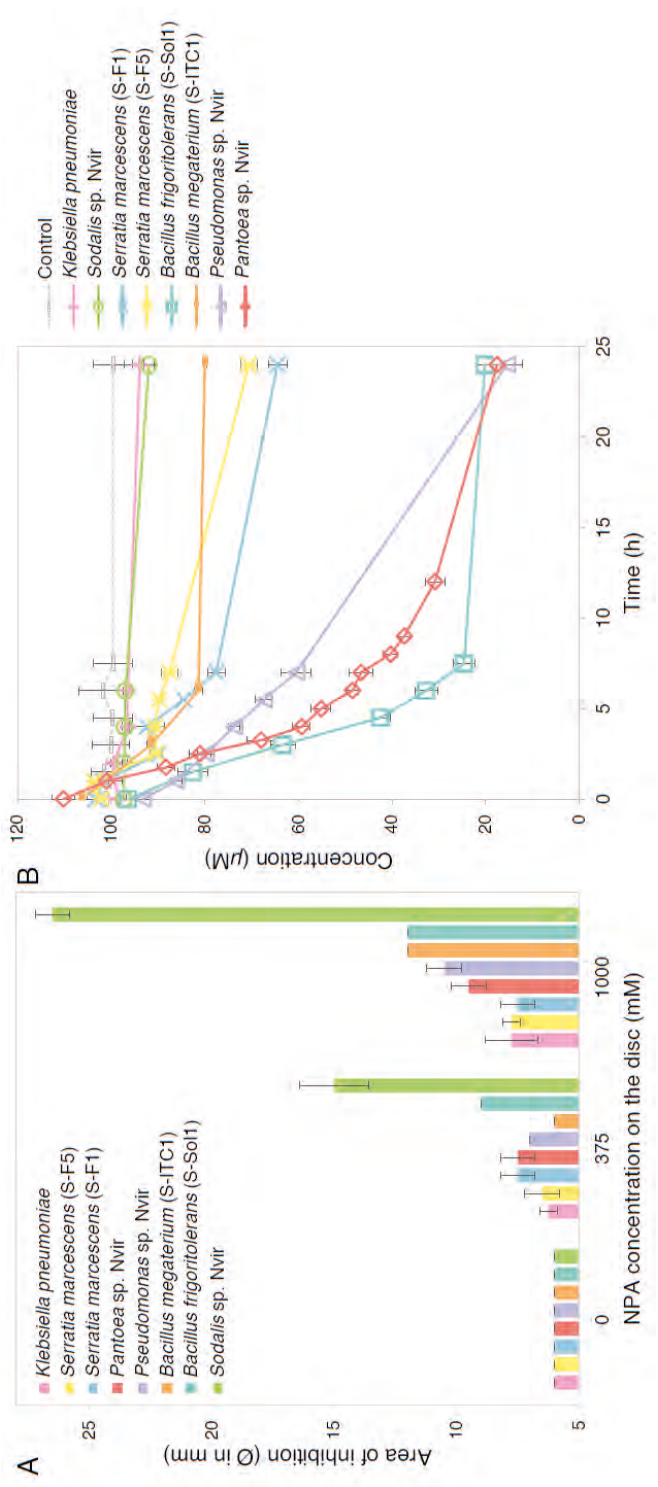

Figure 6.1. Characterization of bacteria isolated from the *N. viridula* microbiome to resist and detoxify 3-nitropropionic acid. A) Bacterial gut isolates were tested for their resistance to NPA in disc diffusion assays with 10 µL of increasing NPA concentration (0 – 1000 mM) applied to the paper discs (6 mm Ø). The diameter of the inhibition zone is expressed in mm ± standard deviation (biological duplicates). The minimal experimental area of inhibition is 6 mm, which is due to the paper disc diameter of 6 mm. **B)** Bacterial gut isolates were incubated in 50 mL M9 mineral salt medium supplemented with 100 µM NPA and detoxification was monitored for 24 hours. Control represents uninoculated M9 medium containing NPA. NPA concentrations were measured by HPLC. Data are represented as mean ± standard error (biological triplicates).

NPA influences amino acid metabolism and transport in *Pantoea*

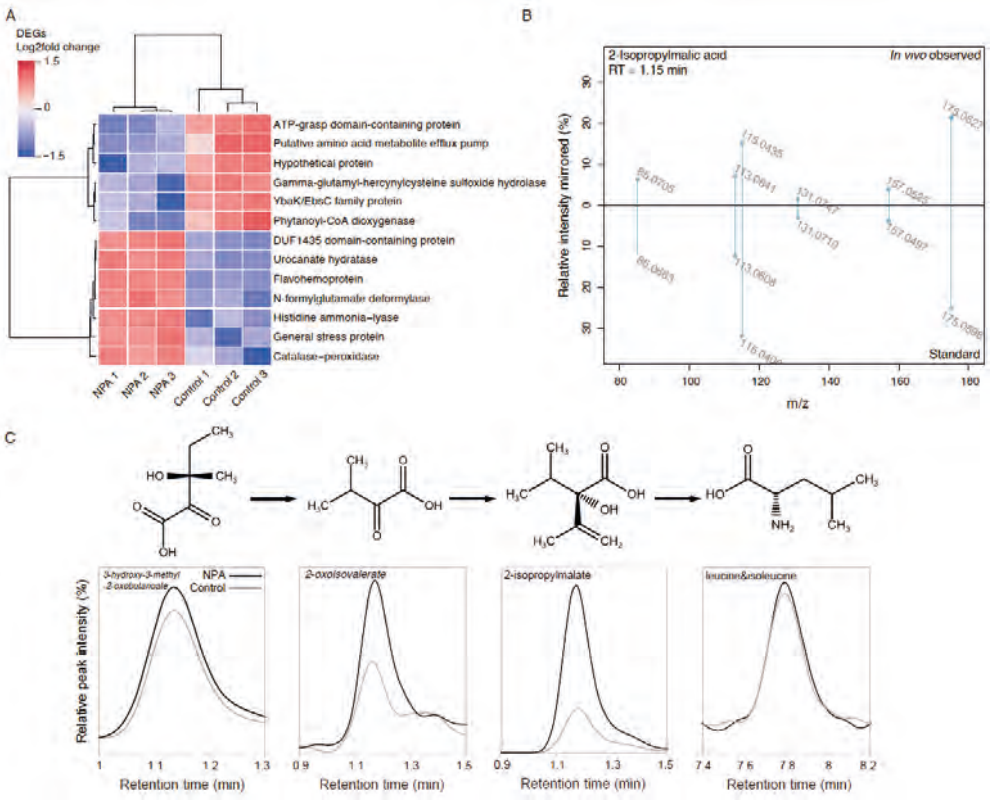

Figure 6.2. Transcriptome and metabolome of *Pantoea* sp. Nvir growing on M9 mineral medium with 100 µM NPA. A) *Pantoea* sp. Nvir differential gene expression profile under growth with and without NPA. *Pantoea* was cultured in an M9 mineral salt medium supplemented with 100 µM NPA or an equimolar concentration of glycerol (control). Samples for RNA extraction (biological triplicates) were taken 2 hours after NPA/glycerol supplementation. A heatmap shows differential gene expression (log_2 fold changes; cutoff = 2; $p < 0.05$) in *Pantoea* cultures. **B)** Mirror plot of observed MS2 feature (top), and MS2 of a commercially purchased 2-isopropylmalic acid standard (bottom) analysed under the same experimental parameters with LC-qToF-MS. **C)** Simplified L-leucine biosynthesis pathway with chemical structures and corresponding chromatograms at 6 hours after NPA or glycerol (control) supplementation. 3-hydroxy-3-methyl-2-oxobutanoate and 2-oxoisovalerate were putatively identified based on m/z value (in italics), whereas 2-isopropylmalate and leucine/isoleucine were identified using chemical standards. Data are represented as the mean of biological quadruplicates.

To further investigate the influence of NPA on bacteria, we analysed the transcriptome of NPA-degrading *Pantoea* sp. Nvir, an obligate symbiont of *N. viridula* (Figure 6.2A). Two hours after the addition of NPA, when NPA was rapidly degraded (Figure 6.1B, Supplementary Figure S4), stress-response genes in *Pantoea* were differentially expressed, demonstrating its toxicity to the cell. Furthermore, NPA seemed to have caused oxidative stress in *Pantoea*, since genes encoding gamma-glutamyl-hercynylcysteine sulfoxide hydrolase and phytanoyl-CoA dioxygenase were downregulated. In mycobacteria, the former is involved in the biosynthesis of the antioxidant metabolite ergothioneine and the latter is associated with a large diversity of oxidative reactions (Hausinger, 2015). Interestingly, all downregulated genes belong to a six-gene-long operon (genes 00010-00015). On the contrary, flavohemoprotein and catalase-peroxidase genes, encoding enzymes involved in NPA breakdown, were upregulated, which supports the observed NPA degradation *in vitro*. Surprisingly, no differential gene expression of the P3N monooxygenase gene was seen. The analysis showed that *pno*A was in fact expressed in both NPA and control samples (data not shown), suggesting that it might be constitutively expressed. Besides this, NPA influenced the expression of genes involved in amino acid metabolism and transport. Genes involved in the metabolism of histidine, encoding histidine ammonia-lyase and N-formylglutamate deformylase, were differentially expressed in cultures exposed to NPA. In contrast, the gene encoding YbaK/EbsC, a protein assumed to prevent translation by blocking the addition of an amino acid to a tRNA molecule, was downregulated in cultures exposed to NPA. The same was true for genes encoding a putative amino acid efflux pump, suggesting inhibition of amino acid biosynthesis and transport and thus possibly impaired supplementation of amino acids for the host.

In a proceeding experiment, we analysed the effect of NPA on the metabolome. Although NPA was rapidly degraded (Figure 6.1B), no accumulation of succinate was observed in *Pantoea*, suggesting that NPA did not inhibit succinate dehydrogenase as suggested for eukaryotes (Bendiksen Skogvold et al., 2022). A similar observation was made in *Sodalis* cultures, which are not capable of NPA degradation (data not shown). Untargeted metabolomics did, however, reveal the accumulation of a metabolite with a m/z of 176.069 $[M+H]^+$ in *Pantoea* cultures exposed to NPA. To identify this metabolite, we collected MS2 fragmentation spectra. When queried against metabolomics databases, the accumulating metabolite was putatively annotated as 2-isopropylmalate (2-IM). Subsequent comparison with a reference compound confirmed the identity of m/z 176.069 as 2-isopropylmalate (Figure 6.2B). 2-IM is an intermediate in the biosynthesis

pathway of L-leucine, in which it is synthesized from 2-oxoisovalerate by 2-isopropylmalate synthase and converted to 3-isopropylmalate by 3-isopropylmalate dehydratase. Ultimately, L-leucine is formed through the activities of 3-isopropylmalate dehydrogenase and leucine transaminase. Figure 6.2C depicts a simplified L-leucine biosynthesis pathway in which we show that the concentration of intermediate metabolites, preceding the formation of L-leucine: 3-hydroxy-3-methyl-2-oxobutanoate (HMO), 2-oxoisovalerate (2-OV) and 2-IM increased in *Pantoea* cells during culturing with NPA. This suggests that NPA has an inhibitory effect on 3-isopropylmalate dehydratase, dehydrogenase, or leucine transaminase, impairing the transformation of intermediate metabolites and L-leucine formation. Nevertheless, the L-leucine concentration in NPA and control samples were identical, with the constraint that leucine and isoleucine are isomers and were not chromatographically separated in our system. In conclusion, both the transcriptome and metabolome of *Pantoea* suggest that NPA has an inhibitory effect on amino acid metabolism and transport, but further analysis of the effect of NPA on other bacteria is necessary to validate this hypothesis. Altogether, this shows that NPA, if not detoxified by other microbial community members, might impair amino acid supplementation by *Pantoea* to the host.

Core microbiota inhibits the growth of transient bacteria

As a phloem feeder, *N. viridula*'s diet is nutritionally imbalanced. Phloem sap (PS) is rich in sucrose, which is the main sugar in PS (Amiard et al., 2004), and poor in amino acids and vitamins. Therefore, insects rely on their symbiotic microbial partners to supplement them with nutrients (Feng et al., 2019; Salem et al., 2014). Likewise, microbes that live in consortia to sustain growth, might depend on each other by biosynthesizing and exchanging certain metabolites (Zhang et al., 2018). Thus, we questioned whether any microbe-microbe interactions between members of the *N. viridula* gut community could lead to synergistic interactions. We determined that all strains favoured growth at 10% sucrose PS medium (Supplementary Figure S4). Depending on the plant species, the sucrose concentration varies considerably, however typically ranges from 10 to 30% (Broussard et al., 2023). Therefore, the artificially created PS medium reflected naturally present conditions and could be used to evaluate microbe-microbe interactions of gut bacteria *in vitro* and shed light on whether diet could shape gut microbial community composition.

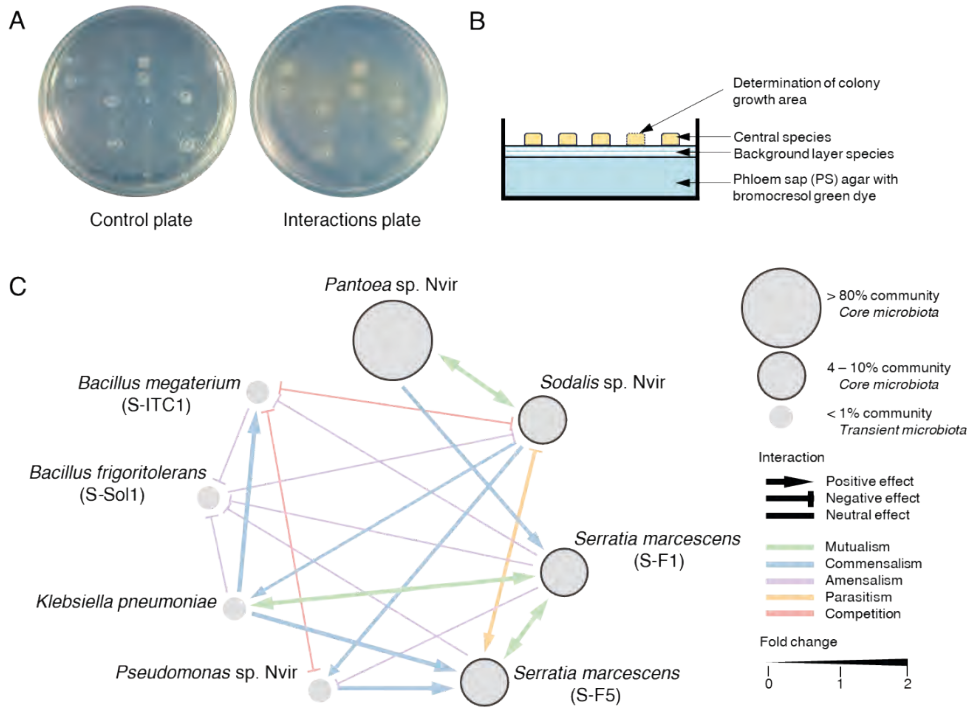

Figure 6.3. Interactions between *N. viridula* gut microbial community members on phloem sap agar. A) Monoculture control plate (no background layer species) and co-culture binary interaction plate with background and central species. **B)** Schematic representation of the method used to detect microbe-microbe interactions on PS medium. Each species was plated as a background layer on 10% PS medium containing bromocresol green dye and all central species were pinned on top. **C)** Interaction network of core and transient gut microbiota of *N. viridula*. Node sizes represent the abundance of microbes in the gut; interactions are indicated with lines ended with arrows (positive effect), blunt arrows (negative effect), or lines (neutral effect); colour indicates the type of the relationship – mutualism (green), commensalism (blue), amensalism (purple), parasitism (orange) and competition (red); line thickness reflects the strength of the interaction expressed as fold change calculated based on the difference of the growth area between interaction plate and control plate.

Next, we set out to identify pairwise interactions between gut species by comparing the performance of the co-cultures against monocultures to identify interactions. Each strain (background layer species) was plated on a 10% sucrose PS agar as a lawn while all strains were pinned on top (central species). Changes in the colony size relative to the background layer were used to determine microbe-microbe interactions (Figure 6.3A-B) (Blasche et al., 2021). The analysis revealed a dense network of interactions between bacteria belonging to the core and transient microbiota (Figure 6.3C), which included

synergistic, negative, and neutral interactions. Remarkably, members of the core microbiota had either mutualistic or commensal relationship, since *Pantoea* and *Sodalis* and both *Serratia* seemed to stimulate each other's growth and *Pantoea* promoted the growth of *S. marcescens* (S-F1). The only negative interactions in the core microbiota happened between *Sodalis* and *S. marcescens* (S-F5), where *Sodalis*' growth was inhibited by *Serratia*. On the contrary, a substantial number of negative interactions were observed between the members of the transient and core microbiota. *Sodalis, S. marcescens* (S-F1), and *Pseudomonas* significantly inhibited the growth of *B. megaterium* while two *Serratia* strains, *Klebsiella* and *B. megaterium* negatively influenced *B. frigoritolerans*. *Klebsiella* is a transient bacterium whose growth was not negatively affected by either microbe but simultaneously contributed to promoting the growth of core and transient microbiota members. A total of five negative interactions were observed between core and transient bacteria, with four positive ones, out of which three were directed towards promoting the growth of *Serratia* strains. With this, we demonstrated that gut bacteria have complex relationships and interact with each other by possible exchange of metabolites, leading to the collaboration between core microbiota and competition between transient bacteria. Moreover, members of the core microbiota suppressed the growth of transient microbes, suggesting that microbes contribute to shaping the insect's gut microbial community, and thus explain the prevalence of certain microbes in the gut.

Change of diet results in microbial community shift in the gut

To ascertain whether diet influences the *N. viridula* gut microbial community composition, the microbiota of the native shield bug population was first determined by 16S rRNA gene profiling (Figure 6.4). The results revealed the dominance of *Pantoea*, *Sodalis*, *Enterococcus*, *Serratia* and *Commensalibacter* in the gut with the abundance of a particular genus differing between individual insects. Transfer of insects from the native population to single-plant diets was however only possible with black mustard and black nightshade. Insects did not establish a colony while feeding on crown vetch solely.

Switching to a single-plant diet as compared to a polyphagous diet resulted in a shift in the microbial community composition. In the *N. viridula* population reared on black mustard, the abundance of *Pantoea* and *Commensalibacter* in the gut microbial community increased in comparison to *N. viridula* grown with a multitude of plants. The *N. viridula* population feeding from black nightshade, in contrast, showed an increased relative abundance of *Sodalis*. Our genomic

analysis indicated that *Sodalis* harboured genes encoding β-glucosidase and β-galactosidase and might therefore be capable of mediating partial degradation of α-solanine (Hennessy et al., 2020). Interestingly, analysis of the plant microbiome indicated the presence of a highly abundant *Streptomyces* in black nightshade, known for its potent antibiotic production properties (Alam et al., 2022). Consequently, this presence could have led to the growth inhibition of various species within the *N. viridula* gut causing the shift of the gut microbial community. Moreover, the results demonstrated that the plant microbiome was quite different from the gut microbial community profile in any insect population, indicating that diet switch does not result in the acquisition of transient microbes from the host plant, but rather in a shift in the abundance of already residing core symbionts.

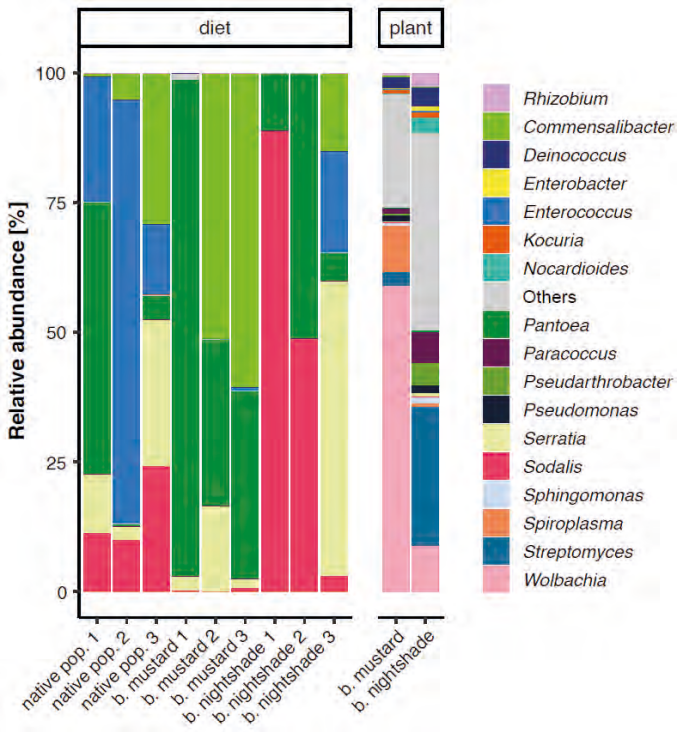

Figure 6.4. Influence of diet change on *Nezara viridula* gut microbial community composition. The samples were compared based on the 16S rRNA gene amplicon sequencing reads between adult *N. viridula* individuals (control; native population) feeding from black mustard, black nightshade, crown vetch, seeds of soybean, sunflower and black mustard, flat beans, and two populations which were fed for six months exclusively either on black mustard or black nightshade. Additionally, the plant microbiome of the plants was taken along as a control. The taxonomy is displayed at the genus level. Others represent the amplicon sequence variants (ASVs) that average below 0.5% of all reads.

Overall, our findings indicate that the gut microbial community of *N. viridula* is relatively stable upon a change in dietary conditions which aligns with the earlier observations of the mutualistic interactions between core microbiota members and repression of transient bacteria. Taken that black mustard and black nightshade belong to distinct plant families and synthesize different secondary plant metabolites to deter insects (Chowanski et al., 2016; Fahey et al., 2001), *N. viridula* might favour symbionts through which detoxifying symbiosis could protect it against toxins or better fulfil nutritional requirements. In summary, regardless of the transition in diet, *N. viridula* maintains beneficial symbiotic microbes in the gut which likely fulfil detoxifying, digestive and nutritional functions.

Discussion

In this study, we investigated the potential of the pest insect *N. viridula*'s core and transient microbiota to detoxify NPA using genomics, transcriptomics, metabolomics, and culturing approaches, and assessed how the host diet and microbes contribute to shaping insect gut microbial community. Endosymbiotic microorganisms associated with insects commonly contain a reduced genome when compared to their free-living counterparts (Hosokawa et al., 2006; Kikuchi et al., 2009; Manzano-Marin & Latorre, 2016; Manzano-Marin et al., 2015). Interestingly, the genomes that were assessed here were obtained from symbionts that were capable of a free-living lifestyle and do not seem to have undergone a substantial genome reduction. Moreover, the genome presented here for the free-living *Pantoea* symbiont differs in size compared to the previously characterized *Pantoea* associated with *N. viridula* (Prado et al., 2009; Tada et al., 2011). Our previous study suggests that in fact several *Pantoea* symbionts in high abundance can be found in the *N. viridula* microbiota (Coolen, Rogowska-van der Molen et al., *under review*) and further studies are required to disentangle their individual roles. The genome analysis of *N. viridula*-associated microbes revealed both host-specific adaptations, such as the ability to biosynthesize essential nutrients, as well as the adaptation to a free-living lifestyle by maintaining complete flagellar assembly (Yun et al., 2014). On the other hand, core and transient microbiota of *N. viridula* harboured large plasmids which could allow bacteria and potentially their host to adapt to changing environmental conditions upon a diet switch to improve nutrient metabolism. Interestingly, as shown in another arthropod endosymbiont *Cardinium*, plasmids might also participate in the ongoing chromosomal genome

reduction (Xiong et al., 2023), and thereby the presence of large plasmids might reflect the beginning of an ongoing genome reduction among *N. viridula* microbial community members (Campbell et al., 2017; Campbell et al., 2015; Van Leuven et al., 2014).

Considering detoxification capabilities, genes encoding for enzymes involved in the degradation of NPA were widespread among core and transient microbial community members. This was in accordance with the observed biodegradation capacity of six out of eight *N. viridula* microbial community members which could benefit the host and non-detoxifying microbial community members with protection against this toxic secondary plant metabolite. Transient microbes, however, grew poorly in co-cultures, and therefore it is unlikely that they benefit *N. viridula* with their NPA degradation capacity. In light of the development of novel pest management strategies, this knowledge could be used to target toxin-degrading microbes via the elimination of the detoxifying genes with the CRISPR-Cas9 mechanism which would decrease the spread of toxin resistance phenotype among insects (Rogowska-van der Molen et al., 2023; Sander & Joung, 2014; Selle & Barrangou, 2015; Zhao et al., 2020). Furthermore, NPA was previously shown to cause irreversible inhibition of the succinate dehydrogenase enzyme in the TCA cycle in eukaryotes and thus accumulation of succinate (Bendiksen Skogvold et al., 2022; Nishino et al., 2010; Rogowska-van der Molen et al., 2022), but a similar observation was not made in the prokaryotes *Pantoea* or *Sodalis*, demonstrating that NPA does not impair the functioning of the TCA cycle in bacteria but rather affects amino acids metabolism and transport. Particularly L-leucine biosynthesis seemed to be inhibited by NPA since it caused intracellular accumulation of L-leucine biosynthesis intermediates. Whether the accumulation of intermediate metabolites caused toxicity to the cells requires further validation.

Members of the microbial community found in *N. viridula* were also shown to interact with one another, shaping the gut microbiota. We unravelled microbial interactions between *N. viridula* gut microbiota members and demonstrated that in an artificial PS medium bacteria formed a complex community network. A clear relationship was observed between two major *N. viridula* symbionts, *Sodalis* and *Pantoea,* which had a strong mutualistic relationship and promoted each other's growth in the sugar-rich and nutrient-deficient PS medium. A similar tendency was shown in facultative *Serratia* symbionts. These findings suggest a metabolite exchange via cross-feeding between bacteria and indicate the adaptations of core microbes to living in proximity (Blasche et al., 2021;

Blasche et al., 2017; Goldford et al., 2018). While core microbiota thrived in the PS medium, the growth of bacteria associated with the transient microbiota was largely inhibited by either transient or core microbiota implying a lack of adaptive ability to sustain growth under realistically nutrient-deficient conditions (Yun et al., 2014). Altogether, our results indicate that both diet and microbial interactions contribute to shaping the host-associated microbial community and with that also its detoxifying symbiosis potential. This dynamic between *N. viridula* microbiota members demonstrated the colonization resistance of core microbes against non-symbiotic bacteria, preventing the disturbance of the gut community.

Diet is another important factor shaping and influencing an insect's phenotype and gut bacterial community (Amato et al., 2019; Colman et al., 2012; Luo et al., 2021). Our results demonstrate that switching from a polyphagous to a single-plant diet caused a shift in core microbial community composition and underscored the dissimilarity to the plant microbiota. These findings imply an overall stability of the core gut microbiome in response to dietary shifts. Likewise, similar patterns were reported by Medina et al. (2018) who observed a shift in the relative abundance of *Pantoea*, *Yokenella* and *Enterococcus*, belonging to the core microbiota, in *N. viridula* during the transition between host plants. Tinker and Ottesen (2016) investigating the gut microbiota of the American cockroach *Periplaneta americana* also found a highly stable core microbial community in response to diet change. The presence of a consistent core microbiota in *N. viridula* suggests that the symbionts are vital to the hosts' fitness (Sudakaran et al., 2015). Analysis of the metabolic potential of *N. viridula*-associated microbiota confirmed the ability to biosynthesize essential amino acids and B vitamins fulfilling hosts' nutritional requirements. Concordantly, several studies reported that the experimental removal of microbiota by egg-surface sterilization negatively affected *N. viridula* fitness and survival (Prado et al., 2009; Tada et al., 2011). On the other hand, we observed that the plant microbiome differed substantially from the gut microbial community implying the selective passing of microbes and suggesting that the selection mechanism could discriminate the core symbionts from transient, non-symbiotic bacteria (Ohbayashi et al., 2015). One such mechanism could be microbe-microbe interactions as seen in the pairwise interactions experiment which demonstrated the mutualistic interactions between core microbiota members and repression of transient bacteria. On the other hand, as described by Kim et al. (2013) insect midgut epithelia could produce antimicrobial substances and in that way control the selective infection of symbionts to the M4

midgut crypts. Taken together, these findings highlight the complex dynamics behind the microbial colonization of the insect gut tract.

While *N. viridula* feeds on a polyphagous diet, transitioning to a single-plant source could pose challenges for polyphagous insects that lack adaptation for a single-plant species diet (Bernays & Chapman, 2007). We observed that *N. viridula* insects established colonies on black mustard and black nightshade but were not capable of feeding solely with crown vetch. A recent study indicated that feeding on various plants provides insects with a nutritional balance and reduces the intake of toxic compounds from individual plant species (Friedrichs et al., 2022), thus feeding on crown vetch solely might have caused limited nutrient availability as compared to black mustard and black nightshade. While our data demonstrated the ability of *N. viridula* microbiota to degrade NPA, the short food retention time and high concentration of NPA in crown vetch (Al-Snafi, 2016; Coolen et al., 2022) suggest that the microbial population might not have sufficient time to degrade the toxin and protect the host or non-detoxifying symbionts. Nevertheless, when comparing gut microbial composition of black mustard and black nightshade populations an evident shift in gut microbial relative abundance was noted. As previously shown in camellia weevils, this variance could potentially result from the production of distinct toxins by the plants to deter insects (Zhang et al., 2020). Black nightshade is known for the production of solanaceous toxins α-solanine and α-chaconine and the observed increase in *Sodalis* abundance could be linked with its potential capability to degrade toxins (Mohy-ud-Din et al., 2010). The genomic analysis revealed that *Sodalis* encoded β-glucosidase and β-galactosidase which could partially degrade α-solanine. Although *Sodalis* lacked α-rhamnosidase, a stepwise removal of glucose and galactose from α-solanine's sidechains was shown to substantially decrease its toxicity (Jensen, Jacobsen, et al., 2009; Jensen, Strobel, et al., 2009). On the contrary, black mustard produces ITC to deter insects that are liberated by the glucosinolate-myrosinase defence system (Winde & Wittstock, 2011). Even though ITCs are well-studied, the microbial detoxification pathway of most ITCs remains unclear, making it challenging to deduct from our genomic data whether *N. viridula* microbiota has the metabolic potential to facilitate ITC degradation. In summary, the results suggested that regardless of dietary shifts, *N. viridula* can maintain core microbes which in return could mediate detoxification of plant toxins and participate in digestion and nutrients supplementation.

In conclusion, our study revealed that the core microbiota of *N. viridula* exhibits signs of ongoing genome reduction by harbouring large plasmids and host-specific adaptation to symbiotic lifestyles. Gut-associated bacteria had varying resistance to NPA and the obligate *Pantoea* symbiont, as well as facultative *Serratia* strains and other transient bacteria, could rapidly degrade NPA, possibly providing *N. viridula* with resistance to plant defences. We also showed a possible new mechanism of action of NPA in bacteria by demonstrating that cultivating *Pantoea* with NPA blocks the biosynthesis pathway of L-leucine and causes the accumulation of 2-isopropylmalate. Further, our findings revealed that the *N. viridula* microbiota is relatively stable in response to diet change, and that microbe-microbe interactions participate in shaping gut bacterial community composition. Taken together, this research highlights the importance of studying insect-plant-microbe interactions to obtain fundamental knowledge on a tri-trophic level which could lead to the elucidation of sustainable pest management strategies in the future.

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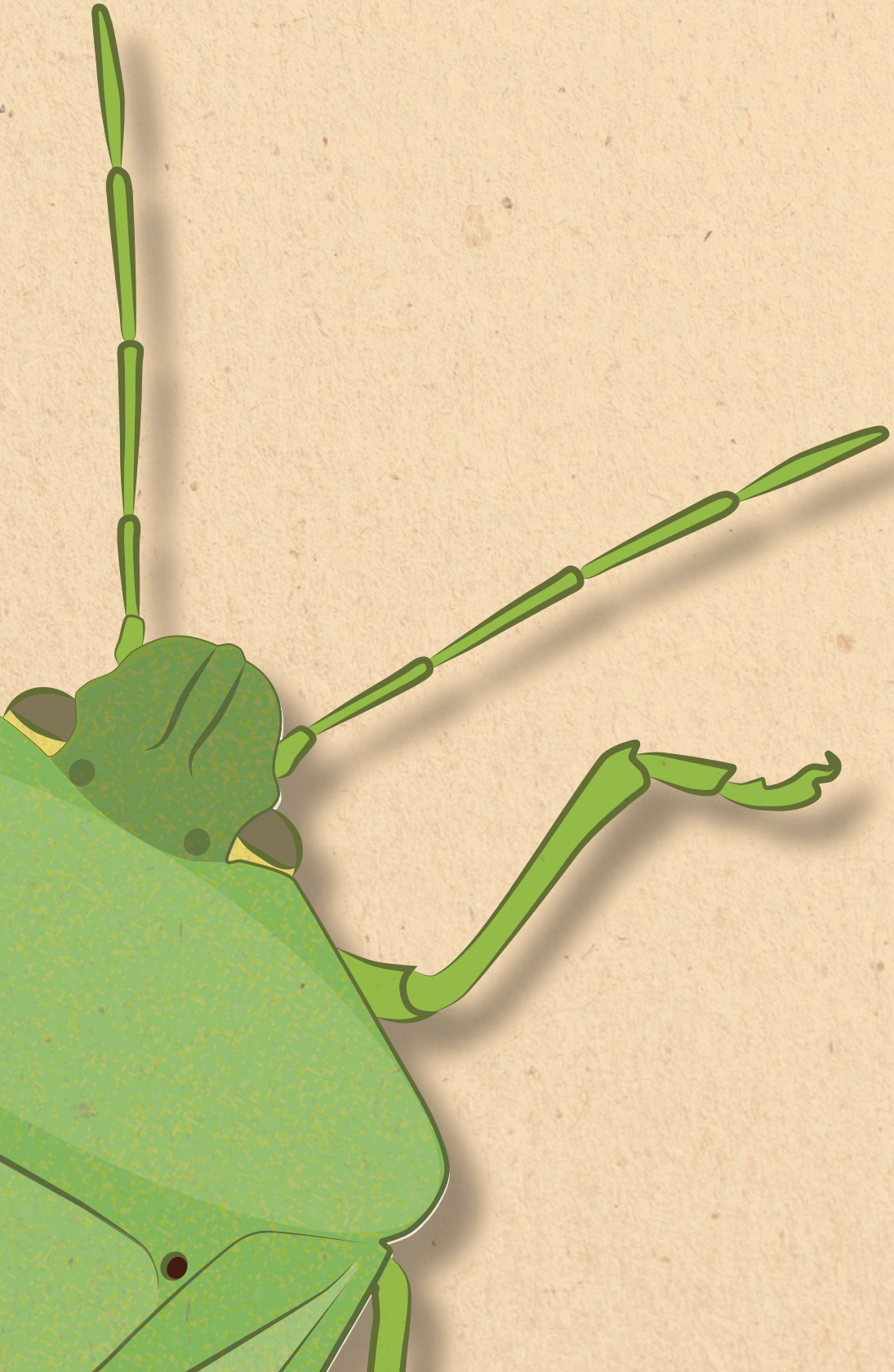

Chapter 7

Insect gut isolate *Pseudomonas* sp. Nvir degrades the toxic plant metabolite nitropropionic acid

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Abstract

Nitropropionic acid (NPA) is a widely distributed naturally occurring nitroaliphatic toxin produced by leguminous plants and fungi. The Southern green shield bug feeds on leguminous plants and shows no symptoms of intoxication. Likewise, its gut-associated microorganisms are subjected to high levels of this toxic compound. In this study, we isolated a bacterium from this insect's gut system, classified as *Pseudomonas* sp. Nvir, that was highly resistant to NPA and was fully degrading it to inorganic nitrogen compounds and carbon dioxide. In order to understand the metabolic fate of NPA, we traced the fate of all atoms of the NPA molecule using isotope tracing experiments with ^{15}N-NPA and 1-^{13}C-NPA, in addition to experiments with uniformly ^{13}C labelled biomass that was used to follow the incorporation of ^{12}C atoms from U-^{12}C-NPA into tricarboxylic acid cycle intermediates. With the help of genomics and transcriptomics we uncovered the isolate's NPA degradation pathway which involves a putative propionate-3-nitronate monooxygenase responsible for the first step of NPA degradation. The discovered protein shares only 32% sequence identity to previously described propionate-3-nitronate monooxygenases. Finally, we advocate that NPA-degrading bacteria might find application in biotechnology and their unique enzymes might be used in biosynthesis, bioremediation and in dealing with postharvest NPA-contamination in economically important products.

Introduction

Approximately 450 representatives within the leguminous plant family (Fabaceae) contain toxic nitroglycosides that can be hydrolysed to 3-nitropropanol (NPOH) and 3-nitropropionic acid (NPA) acting as defence compounds which were found to be exceedingly poisonous to humans and other animals (Anderson et al., 1993). Intoxication by either compound of eukaryotes causes irreversible inhibition of the essential mitochondrial enzyme succinate dehydrogenase in the tricarboxylic acid (TCA) cycle impairing the functioning of the electron transport chain and eventually leading to cell death. Likewise, NPA intoxication leads to the accumulation of toxic nitrite ions which disrupts hemoglobin's oxygen carrying capacity (Al-Snafi, 2016; Anderson et al., 2016; Francis et al., 2013).

In addition to leguminous plants, NPA is produced by many fungi within the *Penicillium* and *Aspergillus* genera (BryanáHanley, 1992). In recent years, fungal contamination with nitroglycosides due to improper postharvest storage and food preparation caused poisonings all over the world leading to serious injuries and even death (Birkelund et al., 2021).

A number of bacteria were reported to degrade toxic nitroglycosides by harbouring nitronate monooxygenase (NMO) encoded in their genomes (Anderson et al., 2000; Nishino et al., 2010). The NMO enzyme converts the conjugate base of NPA to 3-oxopropanoate (3OP) and further to the corresponding semialdehyde releasing nitrite and hydrogen peroxide. Subsequently, 3OP can be converted by a 3OP dehydrogenase to acetyl coenzyme A (acetyl-CoA) resulting in CO_2 generation (Vercammen et al., 2015). Released degradation products such as nitrite are later converted into ammonium, while acetyl-CoA serves as a substrate in the TCA cycle, thereby providing a nitrogen and carbon source for bacteria, respectively (Nishino et al., 2010).

In this study, we investigated the metabolism of an NPA-degrading *Pseudomonas* sp. Nvir isolated from the gut of a pest insect, the Southern green shield bug *Nezara viridula* (Hemiptera: Pentatomidae). With the use of genomics, transcriptomics, and metabolomics we reveal the complete NPA degradation pathway including the metabolic fate of all carbon and nitrogen atoms in the NPA molecule. We discovered that a previously uncharacterized flavin-dependent monooxygenase likely encodes a nitronate monooxygenase, encoded by the *pnm*R gene, with only 32% sequence identity on amino acid level to previously described nitronate monooxygenases, involved in the first step of the degradation

pathway. We advocate that the here isolated *Pseudomonas* sp. Nvir and its unique uncharacterized yet nitronate monooxygenase possess the variety of biotechnological application in agriculture and the food industry as well as in organic synthesis and bioremediation (Torres-Guzman et al., 2021).

Materials and Methods

Insect rearing

Nezara viridula (42 insects, 2nd -4th instar nymphs) were collected in the field from creeping thistle (*Cirsium arvense*) in the Netherlands (51.348028, 6.128802) on the 5th of July, 2019. The insects were transferred to the greenhouse and placed within the rearing cage to establish a colony. *N. viridula* individuals were reared in a greenhouse facility with normal daylights and additional light to obtain a photoperiod of 16:8h (light: dark) year-round. Insects were provided with sunflower (*Helianthus annuus*), soybean (*Glycine max*), and brown mustard (*Brassica juncea*) seeds and native plants of crown vetch (*Securigera varia*), black mustard (*Brassica nigra*) and black nightshade (*Solanum nigrum*).

Bacterial strain isolation

Ten complete gut systems (V1-V4 sections) of adult *N. viridula* were dissected, pooled and mixed in a tube. Gut tissue was disrupted by adding beating beats to a tube and 200 µL of sterile phosphate-buffered solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and brief vortexing. 100 µL of gut suspension was added to 10 mL of M9 mineral salt medium (33.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.55 mM NaCl, 9.35 mM NH_4Cl, 1 mM $MgSO_4$, 0.3 mM $CaCl_2$, Thauer vitamin mixture (Sowers & Ferry, 1985), trace elements (Kurth et al., 2019), pH 7.2) supplemented with 100 µM of NPA (Merck, Germany) as a carbon source. Cultures were grown aerobically in Erlenmeyer flasks (500 mL) at 30°C and 200 rpm. To obtain pure cultures of NPA-degrading bacteria, pre-grown cultures were streaked onto an M9 mineral salt agar plate supplemented with 100 µM NPA. Several colonies were picked and re-streaked three times onto fresh M9 agar plates. Isolated pure cultures were incubated in M9 mineral medium deprived of ammonium chloride, with 100 µM NPA as the sole nitrogen and carbon source, in a similar manner as described above. To define the purity of the cultures which had grown on NPA as a sole nitrogen and carbon source, DNA was extracted and the genomes were sequenced with Mi-Seq Illumina sequencing (Illumina, San Diego, California, United States) as described below.

Genome sequencing and data analysis

DNA was extracted from NPA-degrading pure cultures using the DNeasy PowerSoil Kit (QIAGEN, Germany) according to the manufacturer's protocol, using 1 mL pelleted cell culture lysed with the TissueLyser LT (QIAGEN) for 2 min at 50 Hz. For the genome of the bacterial isolates, the DNA libraries were prepared with the Nextera XT Library Preparation Kit (Illumina, San Diego, California, United States) according to the manufacturer's instructions. The libraries were checked for quality and size distribution using the Agilent 2100 Bioanalyzer and the High sensitivity DNA kit (Agilent Technologies, Santa Clara, California, USA). Quantitation of the libraries was performed by the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc Waltham USA). Paired-end sequencing (2x 300bp) was performed using the Illumina MiSeq sequencer (Illumina, San Diego, California, United States) and the MiSeq Reagent Kit v3 (Illumina) according to the manufacturer's protocol.

The quality of Illumina paired-end genomic sequencing data was assessed using FASTQC 0.11.8 (Bushnell, 2014) before and after quality processing. Quality-trimming, adapter removal, and contaminant filtering of reads were performed using BBDuk (BBTools 38.75; Bushnell, 2014). Trimmed reads were co-assembled *de novo* using MEGAHIT 1.2.9 (Li et al., 2015). MEGAHIT assembled the genome using k-mer sizes of 21, 29, 39, 59, 79, 99, 119, and 141. Reads were mapped back to the genomes using separately BBMAP 38.75 (default settings), Bowtie 2 2.3.5, or Burrows-Wheeler Aligner (BWA MEM) 0.1.17 (Bushnell, 2014; Li & Durbin, 2010). The sequencing mapping files were handled and converted using SAMtools 1.10 (Li et al., 2009). Genome binning was performed for contigs greater than 1500 bp using four binning algorithms: BinSanity 0.3.1 (Graham et al., 2017), CONCOCT 1.1.0 (Alneberg et al., 2014), MaxBin2 2.2.7 (Wu et al., 2016), and MetaBAT 2 2.15 (Kang et al., 2019) using default settings. The bin sets were supplied to DAS Tool 1.1.2 (Sieber et al., 2018) for consensus binning to obtain the final optimised bins. Out of all sequenced reads <100 reads were not mapped to the genome bins. The quality of the generated bins was assessed through a single-copy marker gene analysis CheckM 1.1.2 (Parks et al., 2015). Taxonomic assignment for the trimmed sequencing reads and assembled genomes was performed with Kaiju (Menzel et al., 2016). The genomes were automatically annotated with Prokka 1.13.4 (Seemann, 2014). Genome annotations were examined using Artemis 16.0.0 (Carver et al., 2005). Additional genome information is found in Supplementary Table 1 and represents only features of the *Pseudomonas* sp. Nvir bin.

The analysis of Average Amino acid Identity (AAI) and Average Nucleotide Identity (ANI) of *Pseudomonas* sp. Nvir was executed on http://enve-omics.ce.gatech.edu/, 2021, as described (Goris et al., 2007; Rodriguez-R & Konstantinidis, 2016). AAI and ANI matrices were calculated based on best hits (one-way AAI and ANI) and reciprocal best hits (two-way AAI and ANI) between genomic datasets.

Transcriptome sequencing and data analysis

Prior to RNA extraction, the *Pseudomonas* sp. Nvir culture grew in an M9 mineral salt medium supplemented with glucose and ammonium as described earlier. Upon C/N depletion, NPA was added to treated cultures. Biomass samples were collected at 1.25h after the supplementation of NPA. 1.25h was selected according to the NPA degradation experiment (Figure 4) since it was estimated to account for approximately half of NPA being degraded at that point. Control biomass samples, where no NPA was added, were collected at the same time point. 2 mL samples were centrifuged at 20000 × *g* for 3 min at 4°C and RNA was extracted with the RiboPure™-Bacteria Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Prior to sequencing, bacterial mRNA was purified with the MICROBExpress™ Bacterial mRNA Enrichment Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Transcriptome libraries were constructed using the TruSeq® Stranded mRNA Library Prep protocol (Illumina) according to the manufacturer's instructions. Obtained libraries were checked with Qubit as described before. Equimolar pooled libraries were sequenced using the Illumina MiSeq sequencer (Illumina). For sequencing, the 150 bp sequence chemistry was performed using the MiSeq Reagent Kit v3 (Illumina) according to the manufacturer's protocol in one direction. For both control and treated cultures, RNA was extracted from three independent biological replicates.

The quality of Illumina single-end transcriptome raw reads was assessed with FASTQC 0.11.8 (Bushnell, 2014) prior to read mapping. Single-end reads were mapped against the annotated genome (Prokka 1.13.4) using Kallisto 0.46.2 (Bray et al., 2016) and outputs were summarized with MultiQC 1.11 (Ewels et al., 2016). Read counts were transformed in Tximport (Soneson et al., 2015) for differential gene expression analysis using BioConductor packages in R environment (Huber et al., 2015). To determine differentially expressed genes log2-fold change cut-off of 1 was set. In addition, to address the issue of multiple testing, a false discovery rate (FDR) correction was performed with *a* $P < 0.01$ value using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995).

The differential gene expression was compared between the NPA supplemented cultures and control cultures, where no NPA was added. The heatmaps illustrating differentially expressed gene profiles were constructed in an R environment using heatmap.2 package and displayed as the average of three independent biological replicate experiments.

Cultivation of *Pseudomonas* sp. Nvir and sample collection

The axenic culture of *Pseudomonas* sp. Nvir was pre-grown in batch cultivation (30°C, 200 rpm) with M9 medium containing glucose (20 mM) and ammonium (9.35 mM).. After nitrogen and carbon source depletion, 100 µM of NPA was supplemented. Afterward, 2 × 1 mL of sample (supernatant and cell pellet, obtained by centrifugation at 20000 × *g*, 3 min) were collected for the determination of NPA degradation, glucose, ammonium, nitrite, and nitrate concentrations (supernatant), and culture growth (cell pellet). Supernatant and cell pellet samples were stored at -20°C until analysis. Samples were collected at 0, 2, 4, 6, 8, and 24 hours after NPA addition.

Growth of *Pseudomonas* sp. Nvir

Growth of *Pseudomonas* sp. Nvir on NPA as a carbon, nitrogen and energy source was followed by measuring the optical density at 600 nm (OD_{600}) with a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, USA) for 196h. The experiment was carried out with three independent biological replicates.

Chemical and protein quantification

Nitropropionic acid. High-performance liquid chromatography (HPLC) was performed on an Agilent 1100 system equipped with a diode array detector and a Merck C-18 column (Lichrospher 100 RP-18 endcapped (5µm) column, 250 mm × 4.6 mm). Isocratic analysis was performed with 100% 0.1% ortho-phosphoric acid in water with a flow rate of 1.2 mL min^{-1}. Prior to the analysis, 200 µL of supernatant was acidified with 25 µL 0.1 M sulphuric acid and 100 µL of the sample was injected. NPA was measured at 210 nm with a retention time of 5.2 min.

Glucose. The glucose concentration in the cultures' supernatant was measured with a Glucose Colorimetric/Fluorometric Assay kit (Merck) according to the manufacturer's protocol.

Ammonium. Ammonium concentrations were determined with the ortho-phthaldialdehyde (OPA) method as described (Taylor et al., 1974).

Nitrite and nitrate. To determine the NO_2^- and NO_3^- concentrations the Griess reagent assay was used. 100 µL of supernatant was transferred to a 96-well plate containing 100 µL of Griess reagent (50 µL of reagent A: 1% (w/v) sulfanilic acid in 1 M HCl and 50 µL of reagent B: 0.1% (w/v) naphtylethylene diaminedihydrochloride in water). The plate was incubated for 10 min at room temperature and absorbance was measured at 540 nm with a microplate reader (SpectraMax 190, Molecular Devices, San Jose, California, United States). Next, 27 µL of VCl_3 (10 mg mL^{-1} in 1 M HCl) was added to the sample to reduce nitrate to nitrite. Subsequently, the 96-well plate was then incubated in the dark at 60°C for 30 min and afterward, absorbance was measured at 540 nm. $NaNO_3$ and $NaNO_2$ were used to prepare standard curves.

Protein quantification. Cell growth was determined by measuring the protein concentration of a 200 µL cell pellet with a Pierce BCA Protein Assay Kit (Merck).

Synthesis of labelled ^{15}N-and 1-^{13}C-nitropropionic acid

Stable isotope labelled ^{15}N- and 1-^{13}C-NPA were synthesised according to (Baxter et al., 1992) with modifications. In summary, for ^{15}N-NPA the starting material 3-bromopropionic acid was added to a stirred suspension of sodium ^{15}N-nitrite in dry dimethylformamide (DMF) and the solution was stirred at room temperature for 40 hours. The reaction mixture was diluted with water, adjusted to pH 1 with a 1 M aqueous HCl solution and extracted with diethyl ether.

For 1-^{13}C-NPA, the starting material 3-bromopropanoic-1-^{13}C acid was added to a stirred suspension of sodium nitrite in dry DMF and the solution was stirred at room temperature for 28 hours. The reaction mixture was diluted with water adjusted to pH 1 with a 1 M aqueous HCl solution and extracted with ethyl acetate.

Organic layers of both labelled NPA were washed with brine (saturated NaCl aqueous solution), dried over $MgSO_4$, and evaporated *in vacuo*. The crude product was purified using column chromatography (ethyl acetate: heptane, 0 to 100%). The ^{1}H-NMR and ^{13}C-NMR were recorded at 298K on a Bruker Avance III 400 (400 MHz) or 500 (500 MHz) spectrometers. The detailed synthesis protocol and NMR spectra are included in the Supplement.

Isotope tracing experiments

Isotope tracing experiments with ^{15}N- and 1-^{13}C-NPA were performed in batches with 30 mL M9 medium in 120 mL culture bottles which were sealed with crimp

capped butyl rubber stoppers. In all isotope tracer experiments, 100 µM of NPA was supplied to the cultures. After ^{15}N-NPA supplementation, 500 µL samples were rapidly withdrawn at timepoints 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 5, 6 and 24 hours. In the 1-^{13}C-NPA experiment, samples were only collected at 0 and 24 hour time points to minimize the potential loss of $^{13}CO_2$ caused by frequent sampling. For the reverse isotope tracing experiment with U-^{13}C-isotopically labelled glucose and U-^{12}C-NPA a *Pseudomonas* sp. Nvir culture was pre-grown for 24 hours on U-^{13}C-glucose (Cambridge Isotopes Laboratories, MA, USA) and passaged three times to cultivate highly ^{13}C-enriched biomass. Next, the culture was exposed to U-^{12}C-NPA, and samples were collected at time points 0, 1.25, and 24 hours. All experiments were carried out in three biological replicates and pelleted cell cultures obtained by centrifugation (20000 × *g*, 3 min) were stored at -70°C until further analysis.

Prior to isotope tracing experiments analysis, the extraction of biomass was performed with a mixture of acetonitrile:methanol:H_2O (40:40:20, v:v:v). In short, 200 µL of the 40:40:20 mixture was added to the pelleted cells. Samples were briefly vortexed and incubated for five min on ice. Next, samples were centrifuged (20000 × *g*, 5 min, 4°C) and the supernatant was transferred into a new tube and stored at -70°C until metabolite analysis. Samples were analysed with a 1290 Infinity II LC system coupled to a 6546 Q-ToF MS (Agilent Technologies) (LC/Q-ToF-MS) as described (Jansen et al., 2020). In short, 2 µL sample was injected onto a Diamond Hydride Type C column (Cogent) and separated using a 0.4 mL min^{-1} gradient of water with 0.2% formic acid (A) and acetonitrile with 0.2% formic acid (B). The gradient was as follows: 0–2 min: 85% B, 3–5 min: 80% B, 6–7 min: 75% B, 8-9 min: 70% B, 10–11 min: 50% B, 11–14: 20% B, 14–24: 5% B, followed by 10 min re-equilibration at 85% B. Detection of compounds was performed in the positive and negative ionization modes.

A custom library composed of accurate masses and LC retention times of central carbon and nitrogen metabolites were used to explore the core metabolome of *Pseudomonas* sp. Nvir. The relative abundance of metabolites was determined using Agilent Qualitative Analysis 10.0 and Profinder 10.0 tools. Isotopologue distribution analysis of selected metabolites during isotope tracing experiments was conducted based on the relative isotope intensities. ^{15}N- and 1-^{13}C-NPA samples were corrected for the natural ^{13}C and ^{15}N abundance by Profinder 10.0. This correction was not applicable for the reverse labelling experiment, for which we used uncorrected peak areas.

Gas chromatography-mass spectrometry (GC-MS) analysis of dissolved inorganic carbon (DIC)

Isotopic fractions of DIC in the liquid media were measured based on a modified headspace method (Åberg & Wallin, 2014). 1 mL of liquid culture was collected from the batch incubations with a syringe and directly filtered through a sterile 0.45 µm filter (Whatman, cellulose acetate) and a 26G needle into a vial (12 mL, Exetainer®, Labco Ltd., United Kingdom) containing 340 µL 6M HCl and crimp sealed with a rubber stopper. Prior to adding the liquid sample, vials with HCl were flushed with 100% N_2 gas to void the headspace of background CO_2. Samples were equilibrated with the acid in the bottles for at least one hour at room temperature to drive all DIC into the gas phase. 50 µL of the bottles' headspace was injected into a gas chromatograph (Agilent 6890 equipped with a 6 ft Porapak Q column) coupled to a mass spectrometer (Agilent 5975C MSD; Agilent, Santa Clara, CA) with a gas-tight syringe (Hamilton, Reno, Nevada, United States). The gas chromatograph was set at 80°C with helium as a carrier gas at a flow rate of 24 mL min^{-1} to determine the isotopic fraction of $^{13}CO_2$.

Phylogeny analysis

To determine the distribution of the *pnm*R gene product, the PNMR amino acid sequence was used as a query in a BLASTp search at NCBI (Altschul et al., 1997) that targeted only bacteria (taxid: 2). The hits with an identity percentage >40% and E values of $<1.0 \times e^{-20}$ were considered in the further analysis. Likewise, the phylogeny of the *pnm*R was assessed within the flavin-dependent monooxygenase family, based on the classification (Huijbers et al., 2014). The amino acid sequences for representative proteins within every class were obtained from UniProt (UniProt, 2021). The amino acid sequences were extracted and compared by multiple sequence alignment with MUSCLE v3 8.31 (Edgar, 2004) and phylogenetic trees were calculated with MEGA7 (Kumar et al., 2016). The evolutionary history was inferred with the Neighbor-Joining method with 500 bootstraps.

Results

Isolation and taxonomy of NPA-degrading *Pseudomonas* sp. Nvir

To isolate NPA-degrading bacteria, gut systems of the Southern green shield bug *N. viridula* (Hemiptera: Pentatomidae) were dissected and pooled. To enrich NPA-degrading bacteria, the gut suspension was inoculated in selective M9 mineral salt medium containing NPA as the sole carbon and energy source. In

this attempt, several isolates were obtained and further investigated. Selected strains were subsequently grown in the same medium where NPA served as the sole carbon, nitrogen and energy source. With this approach, only one isolate was further examined, since unlike others it was able to grow exclusively on NPA. We investigated the influence of NPA concentration on the isolate's resistance and observed that it withstood up to 10 mM NPA (data not shown). The full genome of the NPA-degrading isolate was sequenced and had a size of 5,716,865 bp with a G+C content of 62.3%. A total of 5464 putative genes were annotated, with 5320 protein-coding genes (CDS), 70 tRNAs, four rRNA operons, and one tmRNA (Supplementary Table 1). Taxonomic analysis with the CheckM tool showed that the isolate was classified as *Pseudomonas* sp. designated here as Nvir (N*ezara* vir*idula*) (Parks et al., 2015). The comparison of Average Amino acid Identity (AAI) and Average Nucleotide Identity (ANI) of ten *Pseudomonas* sp. genomes revealed that *Pseudomonas* sp. Nvir shared a high similarity (AAI 94% and ANI 88%) with *Pseudomonas putida* and *Pseudomonas monteilii* (Supplementary Figures 1 and 2).

Pseudomonas sp. Nvir growth is supported by NPA

We sought to elucidate the NPA degradation pathway in *Pseudomonas* sp. Nvir, by exploring the growth and capability to use NPA as a carbon, nitrogen and energy source. During the isolation procedure, the strain was grown exclusively on NPA as carbon, nitrogen and energy source, suggesting its ability to mineralize NPA. A growth curve of *Pseudomonas* sp. Nvir with NPA as sole carbon, nitrogen and energy source is shown in Figure 7.1A. We found that the growth of the isolate was not proportional to the increasing NPA concentration. In fact, instead of a 10 fold increase in growth only roughly 40% increase in biomass growth on 1 mM NPA in comparison with 100 µM was observed. The disproportional growth of *Pseudomonas* sp. Nvir might be the explained by the inhibitory influence of nitrite build-up in the beginning stage of NPA degradation as well as toxic effect of the high concentration of NPA. On the other hand, in control cultures 0.001 and 0.0001% ethanol solutions were used, as the original stock of NPA used for these experiments was prepared in ethanol. The growth of control cultures was minimal and no difference was seen between them, indicating that the growth of biomass with supplemented NPA depended entirely on the presence of NPA in the medium.

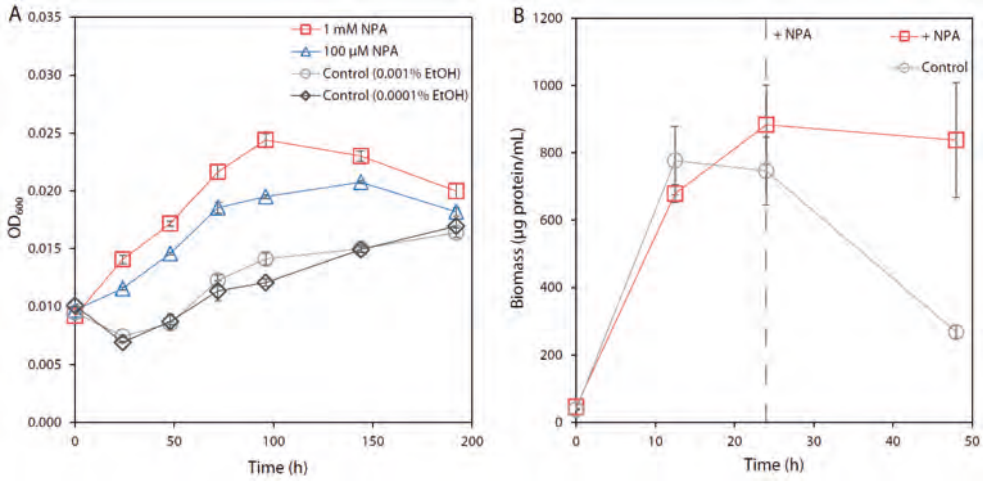


Figure 7.1. A) Growth of *Pseudomonas* sp. Nvir on NPA as sole carbon, nitrogen and energy source. The medium was inoculated with biomass at an OD_{600} of 0.01. The growth was monitored in control cultures and in cultures supplemented with 1 mM NPA or 100 µM NPA by measuring OD_{600} for 196h. As controls, 0.001% and 0.0001% ethanol solutions were used, since the initial stock of NPA was dissolved in 99% ethanol. Data are represented as mean ± standard error (n = 3)**. B)** Cell pellet biomass quantification of *Pseudomonas* sp. Nvir during presence and absence of NPA. The medium was inoculated with approximately 20 µg protein/mL biomass and pre-grown in an M9 mineral medium containing 20 mM of glucose and 9.35 mM of ammonium chloride. Subsequently, *Pseudomonas* sp. Nvir cultures were grown for 24 hours upon both glucose and ammonia were depleted from the medium. After 24 hours 100 µM NPA was added to one culture (dashed line), designated as + NPA, and compared with a control culture, where no NPA was added. The growth of *Pseudomonas* sp. Nvir cultures were monitored over 48 hours by measuring cell pellet biomass protein content using a colorimetric assay. Data are represented as mean ± standard error (n = 3).

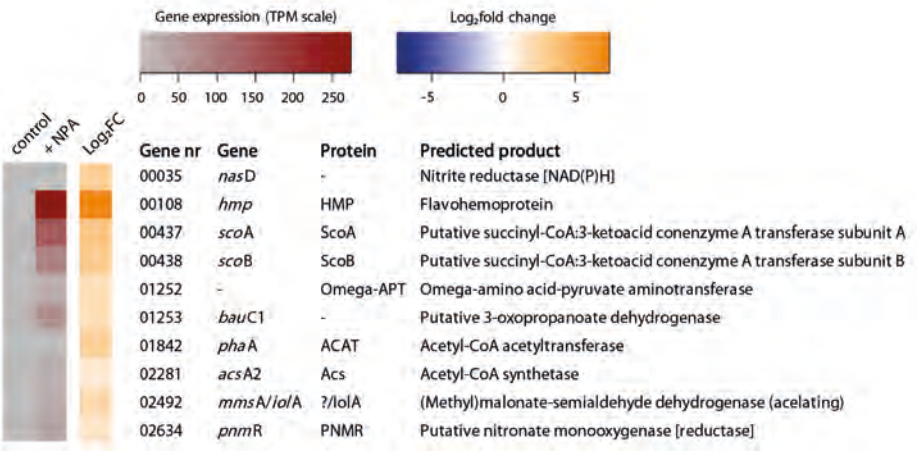
Due to the slow growth and low biomass yield of this isolate on NPA which hampered downstream analyses, we aimed to obtain high cell density cultures in further experiments. For this reason, we simultaneously grew two cultures in M9 mineral salt medium containing glucose and ammonium as sole carbon and nitrogen substrates. With this, we aimed to investigate the influence of NPA on the culture viability. After a 24-hour C/N depletion step (Supplementary Figure 3), we quantified the protein content of the cell pellet biomass in the absence or presence of NPA (Figure 7.1B). The supplementation with 100 µM NPA at the 24-hour timepoint showed that the control culture decreased in biomass rapidly after 24 hours, whereas the addition of NPA resulted in a steady state of biomass. The sudden decrease in the protein quantity might be caused by the cell lysis under nitrogen and carbon depleting conditions. In this experiment,

only the pelleted cells were tested for the protein quantity resulting in declining protein quantity upon biomass lysis caused by the substrate depletion. The slight decrease in NPA supplemented culture might reflect that NPA supported the culture viability, yet was not sufficient to provide growth. Moreover, the lysis likely was caused by the depletion of the growing substrates since, no pH decrease that could cause protein precipitation was seen (data now shown). This data thereby implies that NPA was used for growth under these conditions enabling further experiments with higher biomass requirements.

Transcriptome analysis

To explore the metabolic potential of *Pseudomonas* sp. Nvir in degrading NPA, we performed transcriptome analyses and compared differential gene expression profiles between NPA-free (control) and NPA-supplemented cultures. Both cultures were grown similarly as described in the growth experiment and samples for the RNA extraction were collected after 1.25h of NPA addition, where approximately 50% of the added NPA had been degraded (Figure 7.4). Control culture was sampled during the stationary state reached after growing on glucose and ammonium. Out of 4.8, 4.7 and 4.9M obtained reads, 3.6, 3.8, and 3.8M were mapped in control samples, respectively, whereas, in NPA-treated cultures out of 5.7, 5.6, and 6.0M reads, 4.2, 4.0, and 4.4M were mapped accordingly. Transcriptomic analysis revealed that 54 genes were significantly (Log_2fold change cut-off = 1) up- or downregulated when the culture was exposed to NPA (Supplementary Figure 4), however, only ten genes seemed to be of relevance in terms of NPA degradation (Figure 7.2). The preliminary analysis revealed that upon NPA stress, *Pseudomonas* sp. Nvir expressed genes encoding essential enzymes involved in the conversion of NPA and its intermediates. Unexpectedly, the previously described *NMO* gene and the putative propionate-3-nitronate monooxygenase (encoded by the *pno*A gene) coding for the enzyme priopionate-3-nitronate (P3N) monooxygenase, which were found to be essential in the degradation of NPA, were not found in the genome or were not expressed, respectively (Nishino et al., 2010). In fact, even though the *pno*A gene was present in the genome of *Pseudomonas* sp. Nvir, expression levels were not detectable in the presence or absence of NPA. Comparison of the *pno*A gene found in this study with the homologous gene of the NPA-degrading *Pseudomonas* sp. JS189 showed that those two genes only shared 42% similarity based on amino acid sequence (Nishino et al., 2010). Furthermore, in NPA-degrading *Pseudomonas*, genes involved in the NPA degradation were part of an operon and located in proximity with a transcriptional regulator (Nishino et al., 2010; Vercammen et al., 2015). *pno*A

of *Pseudomonas* sp. Nvir seemed, however, to be randomly located in the genome, which jointly with its low amino acid identity to characterized enzymes might explain the lack of *pno*A expression when exposed to NPA. Since our *Pseudomonas* sp. Nvir isolate was clearly capable of NPA degradation, these results suggested the presence of a hitherto undescribed enzyme(s) for NPA degradation. Subsequent analysis of upregulated genes within the transcriptome resulted in the identification of gene 02634 which belonged to the MSMEG 0569 flavin-dependent oxidoreductase family as classified by National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). Although gene 02634 and *pno*A share only 32% amino acid sequence identity, evolutionarily they belong to the same superfamily. Therefore, we hypothesised that the enzyme encoded by gene 02634 might harbour the same catalytic function as the NMO or P3N monooxygenase. We propose to name gene 02634 found in *Pseudomonas* sp. Nvir as a putative nitronate monooxygenase [reductase] with designated gene and protein abbreviations *pnm*R and PNMR, respectively.



Gene nr	Gene	Protein	Predicted product
00035	*nas*D	-	Nitrite reductase [NAD(P)H]
00108	*hmp*	HMP	Flavohemoprotein
00437	*sco*A	ScoA	Putative succinyl-CoA:3-ketoacid conenzyme A transferase subunit A
00438	*sco*B	ScoB	Putative succinyl-CoA:3-ketoacid conenzyme A transferase subunit B
01252	-	Omega-APT	Omega-amino acid-pyruvate aminotransferase
01253	*bau*C1	-	Putative 3-oxopropanoate dehydrogenase
01842	*pha*A	ACAT	Acetyl-CoA acetyltransferase
02281	*acs*A2	Acs	Acetyl-CoA synthetase
02492	*mms*A/*iol*A	?/IolA	(Methyl)malonate-semialdehyde dehydrogenase (acelating)
02634	*pnm*R	PNMR	Putative nitronate monooxygenase [reductase]

Figure 7.2. *Pseudomonas* sp. Nvir differential gene expression profile under NPA conditions. *Pseudomonas* sp. Nvir cultures grew in an M9 mineral medium supplemented with glucose and ammonium. After 24-hour C/N depletion phase, 100 µM of NPA was added to the culture. Samples for the RNA extraction were collected at 1.25h after NPA supplementation. Likewise, control samples where no NPA was added, were collected at the same time point. A heatmap shows transcript expression level (TPM) under presence (+ NPA) or absence of NPA (control) and Log_2fold changes (Log_2FC) in the gene expression in the ten differentially expressed genes in *Pseudomonas* sp. Nvir. The complete transcriptional profile is provided in Supplementary Figure 4.

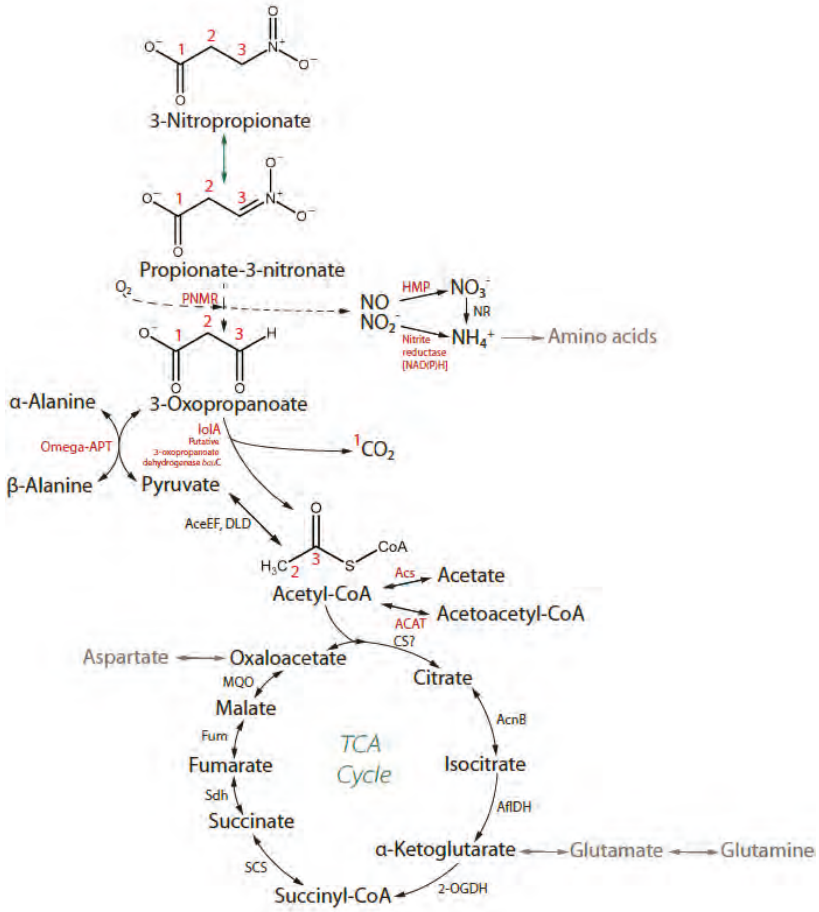


Figure 7.3. Predicted metabolic pathway of NPA degradation constructed based on the analysis of the differentially expressed genes and experimental evidence. A prediction was made on the role of the NPA in the central metabolism. Based on the experimental evidence we showed the biotransformation of 3-nitropropionate to nitrite and nitrate, release of CO_2 and incorporation of C2 product to TCA cycle, by quantifying aspartate, malate, glutamate and succinate. The bioconversion pathway of intermediary products, 3-oxopropanoate and pyruvate, to α- and β-alanine and acetyl-CoA to acetate and acetoacetyl-CoA were hypothesised according the transcriptomic data set and available literature. The activity of PNMR was presumed based experimentally shown accumulation of nitrite and nitrate and its phylogenetic placement within the monooxygenase family. The complete characterization of the enzymatic activity of this enzyme remains yet to be determined. In the predicted metabolic pathway, black arrows represent reactions of NPA degradation, metabolism of generated by-products, and the TCA cycle. A green arrow indicates spontaneous biochemical conversion under physiological pH. According to the literature, grey arrows indicate amino acid biosynthetic pathways. A dashed line indicates a hypothetical pathway of the PNMR. Enzymes highlighted in red indicate encoded designated proteins highly expressed upon NPA exposure. Enzymes, in black, are identified in the genome but not highly expressed and the "?" indicates a protein or pathway not identified in the genome. NR, nitrate reduction to ammonium probably is catalyzed by NasA and NirBD Red numbers represent the flow of carbons based on their original position in NPA.

Furthermore, several genes encoding enzymes participating in either the direct degradation process or the transformation of the generated by-products were upregulated. According to the differentially expressed genes, a complete degradation NPA pathway was reconstructed (Figure 7.3) showing the suggested fate of all atoms in the overall metabolism. Biochemically, NPA at physiological pH exists in equilibrium with its conjugate base, P3N, and both the acidic and anionic form undergo a denitration reaction catalysed by PNMR. NPA and P3N are oxidised to 3-oxopropanoate (3OP) losing their nitro group releasing nitric oxide and nitrite. Nitrite is subsequently reduced by nitrite reductase to ammonium (catalysed by the *nas*D-encoded protein) and nitric oxide is rapidly metabolized into nitrate by a flavohemoprotein (HMP). Finally, nitrate reduction (catalysed by the *nas*A- and *nir*BD-encoded enzymes) yields ammonium, which serves as a nitrogen source for amino acids following nitrogen assimilation. Next, the intermediate product 3OP formed in the first degradation step could serve as a substrate to the α-alanine metabolism, generating β-alanine and pyruvate. Alternatively, 3OP is oxidised by (methyl)malonate-semialdehyde dehydrogenase and/or a putative 3OP dehydrogenase to acetyl-CoA and CO_2. Acetyl-CoA is transformed into several metabolites, such as acetate, acetoacetyl-CoA or enters the TCA cycle where it is further used in the central carbon metabolism. Additionally, we found that the *sco*AB genes were upregulated which would suggest that the corresponding enzyme 3-oxoacid CoA-transferase (ScoAB) synthesized acetoacetyl-CoA from succinyl-CoA, which is a part of the succinyl-CoA degradation pathway (not shown). Overall, the differential expression gene profiles suggest a possible NPA degradation pathway including the subsequent transformation of the majority of the by-products. Following up on this hypothesis, we aimed to confirm the hypothetical degradation pathway using metabolomics and provide more information on PNMR by studying its evolutionary history.

Pseudomonas sp. Nvir degrades NPA and releases nitrite and nitrate

We sought to substantiate the proposed NPA degradation pathway by studying the metabolome of *Pseudomonas* sp. Nvir. Therefore, we aimed to detect metabolic degradation of NPA by the isolate by measuring the concentration of NPA, nitrite, and nitrate. NPA was added to a pre-grown culture, and the concentration of NPA, nitrite and nitrate were monitored over 24 hours (Figure 7.4). Within the first six hours, 100 µM NPA was completely utilized by the culture. As indicated in the transcriptome data, the immediate degradation of

NPA was likely caused by the upregulation of already expressed genes when no NPA was present (control samples). Likewise, the constitutive expression of genes involved in NPA degradation with a conceivable low turnover rate of corresponding enzymes would allow rapid utilization of NPA with simultaneous release of nitrite and nitrate.

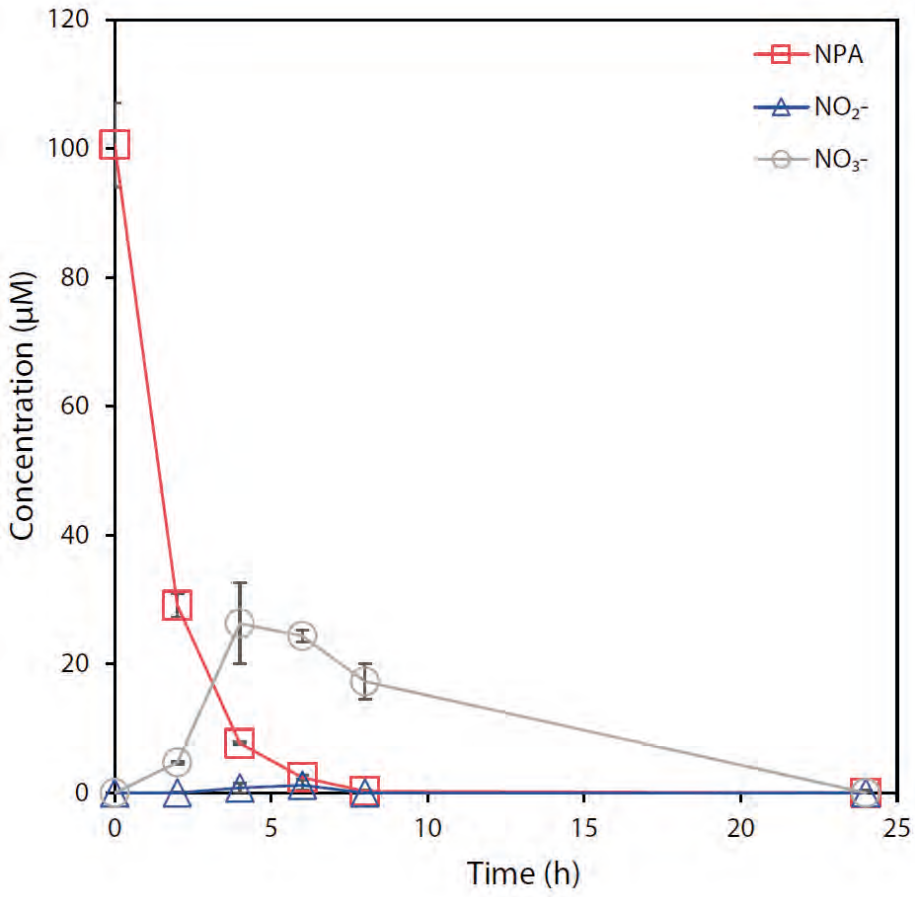


Figure 7.4. NPA, nitrate and nitrite profiles in *Pseudomonas* sp. Nvir cultures. *Pseudomonas* sp. Nvir pre-cultures were first grown for 24 hours in M9 mineral medium with 20 mM glucose and 9.35 mM ammonium chloride as sole carbon and nitrogen source. After the 24-hour-depletion of the primary substrates, 100 µM NPA was added to the cultures and changes in the concentration of NPA, nitrite, and nitrate were monitored. Data are represented as mean ± standard error (n = 3).

With this experiment a steady increase in nitrate and to a lesser degree in nitrite concentration was observed soon after NPA supplementation. Likewise, both nitrite and nitrate concentrations gradually decreased after four hours and were no longer detected at the 24-hour timepoint. As a result, nitrate and nitrite seem to be by-products of the NPA degradation, since in control cultures where no NPA was added, neither nitrate nor nitrite were formed (data not shown). The aforementioned results support the hypothesis that nitrite and nitrate are the products of the proposed NPA-breakdown pathway. In addition, the decrease in nitrate and nitrite levels after four hours suggests that nitrate and nitrite were further transformed and possibly acted as a nitrogen source for *Pseudomonas* sp. Nvir. This observation is in accordance with the earlier observed growth of *Pseudomonas* sp. Nvir in the culture supplemented with NPA.

NPA is used as a nitrogen source

To test the hypothesis that nitrate and nitrite present in the medium during NPA degradation were transformed and subsequently used as nitrogen source, an isotope tracing experiment with ^{15}N-NPA was performed (Figure 7.5A). ^{15}N-NPA was synthesised specifically for this study to determine the metabolic fate of the nitrogen atom from NPA during the breakdown reactions. The introduction of the isotopically labelled NPA into the culture revealed the incorporation of ^{15}N atoms into glutamine and glutamate. Initially, there was only a small increase in the abundance of ^{15}N-glutamine and glutamate which rapidly increased after three hours. A simultaneous increase in nitrite and nitrate concentrations reported earlier supports the evidence that inorganic nitrogen by-products were transformed to ammonia and subsequently used in the biosynthesis of amino acids. After 24 hours, approximately 15% of glutamate and 8% of glutamine carried ^{15}N atoms. Since glutamine and glutamate are the main nitrogen donating metabolites, these results indicate that NPA acts as a nitrogen source.

CO_2 is the by-product of NPA degradation

To confirm that CO_2 was generated during the degradation of NPA, we performed a tracing experiment with 1-^{13}C-NPA. The results indicated that there was a significant increase in $^{13}CO_2$ concentration after a 24-hour period (Figure 7.5B). No change in the abundance of $^{13}CO_2$ was seen in the control. The initial concentration of $^{13}CO_2$ in both treated and control samples represents the natural abundance of $^{13}CO_2$ in the air. Likewise, the incorporation of ^{13}C atoms was not found in amino acids or TCA metabolites, suggesting that CO_2 is a direct product of NPA degradation. With this experiment, we showed that the carboxyl group was removed from the NPA which resulted in the release of CO_2.

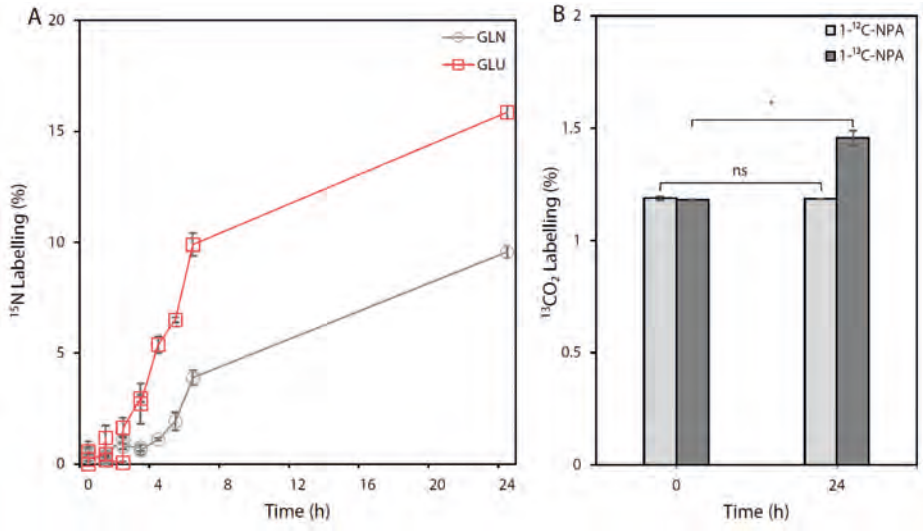


Figure 7.5. The metabolic fate of ^{15}N and ^{13}C atoms from the NPA during isotope tracer experiments in *Pseudomonas* sp. Nvir cultures. A) ^{15}N-enrichment of glutamine (GLN) and glutamate (GLU) during ^{15}N-NPA tracing experiment. The graph represents the sum of all labelled fractions. **B)** $^{13}CO_2$ enrichment during 1-^{13}C-NPA tracing experiment. As control 1-^{12}C-NPA was used. Significance was tested with a Student's t-test for paired samples and accepted at $*P < 0.05$ ($n = 3$); non-significant associations are indicated with "ns". All measured metabolites represent the average of three independent biological replicate experiments ± standard error.

NPA intermediates enter central carbon metabolism

Ultimately, if our proposed pathway was correct, labelled acetyl-CoA should enter the TCA cycle. To confirm this, we analysed the labelling profiles of selected TCA cycle metabolites. To do so, we first pre-grew *Pseudomonas* sp. Nvir cultures in U-^{13}C-glucose to uniformly label all metabolites with ^{13}C. Next, we fed the biomass with U-^{12}C-NPA and traced the incorporation of ^{12}C atoms into succinate, malate, aspartate, and glutamate (Figure 7.6). Although aspartate and glutamate are not part of the TCA cycle, they are considered to be in equilibrium with oxaloacetate and αketoglutarate, respectively, and therefore mirror their ^{12}C labelling. Moreover, we monitored the ^{12}C incorporation by comparing the abundance of metabolites in the beginning of the experiment (0h), after 1.25 hours and after 24 hours. 1.25 hours was selected according to the NPA degradation experiment (Figure 7.4) since it was estimated to account for approximately half of NPA being degraded at that point. In this experiment, we looked at the profiles of individual fractions and compared their changes over time.

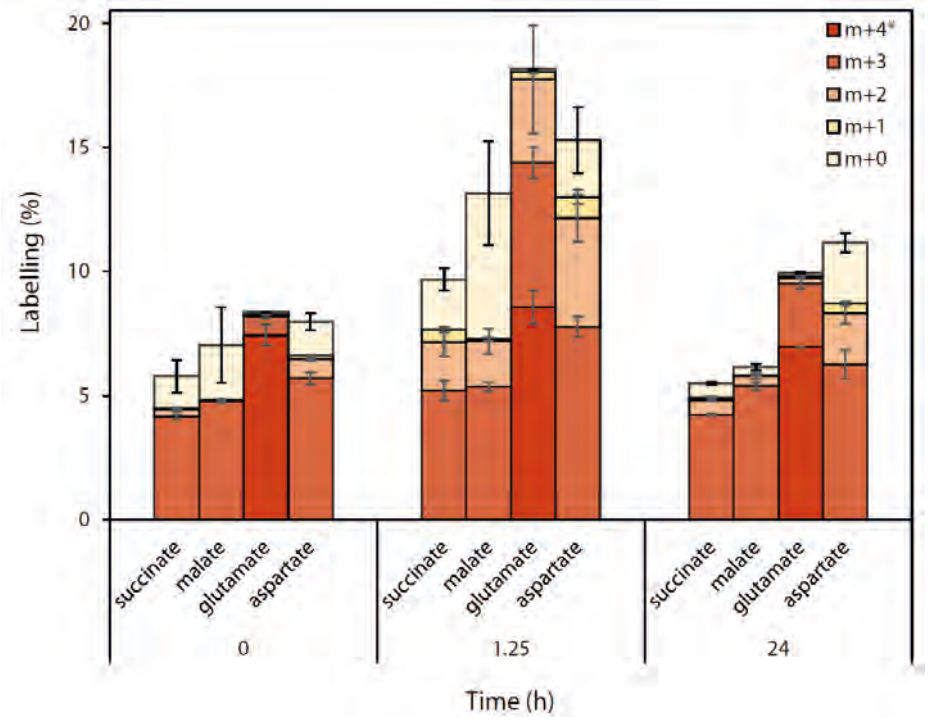


Figure 7.6. Elucidating the TCA cycle of *Pseudomonas* sp. Nvir with U-^{13}C-glucose. Time-series of the isotope distributions of selected TCA cycle metabolites during isotope tracing experiments. Cells were grown with U-^{13}C-glucose and received a pulse of U-^{12}C-NPA at t=0. The m+4 fraction is only shown for glutamate. The m+5 fraction of glutamate and m+4 fractions of succinate, malate, and aspartate represented the maximum level of labelling would obscure the change of the ^{13}C label and are therefore not displayed. The full dataset can be found in Supplementary Figure 5. All measured metabolites represent the average of three biological replicates ± standard errors.

We hypothesised that two ^{12}C atoms from the NPA and correspondingly from the degradation product acetyl-CoA entered the TCA cycle. In accordance with this hypothesis, at 1.25h an increase in m+2 and m+0 fractions of succinate, malate, and aspartate was observed, with only little change in abundance of the m+1 fraction. Accordingly, this means that indeed two and four ^{12}C carbons, respectively, from acetyl-CoA were incorporated into succinate, malate, and aspartate, generating less-labelled fractions of aforementioned metabolites. A similar pattern of ^{12}C incorporation into glutamate was observed, as illustrated by the increase in the abundance of the m+3 and m+2 fractions. Correspondingly, the abundance of all fully-labelled fractions of examined metabolites decreased proportionally at 1.25h.

After 24 hours, due to the extensive scrambling of the introduced ^{12}C, the abundance of unlabelled fractions of all metabolites decreased and resembled the initial stage. Based on this phenomenon, it seems that ^{12}C atoms were first incorporated into the TCA cycle and subsequently, as a result of the metabolite flux, redistributed into various metabolic pathways resulting in an increase in the abundance of fully-labelled fractions. These results evidently indicate that the ^{12}C from U-^{12}C-NPA was indeed incorporated into the central metabolism and that NPA served as a carbon source.

*pnm*R is widely distributed among bacteria

We confirmed that *Pseudomonas* sp. Nvir degrades NPA and according to the isolate's metabolome, genome, and transcriptome analysis, we established its NPA degradation pathway. To obtain more insight into the distribution of the here investigated NPA degradation pathway, the phylogeny of PNMR was investigated by performing multiple sequence alignments with proteins clustered within the flavin-dependent monooxygenase family. This protein family is divided into eight groups according to the classification proposed by Huijbers *et al.* (Huijbers et al., 2014). The PNMR amino acid sequence was aligned with representatives of each group and the similarity was assessed according to the generated alignment. The results showed that PNMR possesses only <63% amino acid similarity to representatives of any of these eight groups (data not shown). *Pseudomonas* sp. Nvir also contained a classical *pno*A gene encoding for a P3N monooxygenase that clustered with the previously well-characterized *Pseudomonas* sp. JS189. This gene was not expressed under our experimental conditions and was therefore not responsible for the NPA degradation. Interestingly, PNMR did not cluster with P3N monooxygenase and is therefore evolutionarily distinct, with low sequence similarity (32%). We, therefore, aimed to assess the distribution of *pnm*R in bacteria by mining the corresponding amino acid sequence in the public database. PNMR as a member of the flavin-dependent oxidoreductase MSMEG 0569 family was found in some Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Cyanobacteria, and bacteria with high G+C content (with minimal amino acid sequence similarity of 40%). The phylogenetic analysis showed that within Gammaproteobacteria, PNMR seems to be restricted to the genus *Pseudomonas* (Figure 7.7). Concluding, the analysis demonstrates the low degree of sequence identity of PNMR with other characterized enzymes, in particular P3N monooxygenase, within the flavin-dependent monooxygenase family, and establishes its evolutionary distinct position within this family. Furthermore, we show that the *pnm*R gene is widely distributed in many bacteria, suggesting that various bacteria may be capable of NPA degradation.

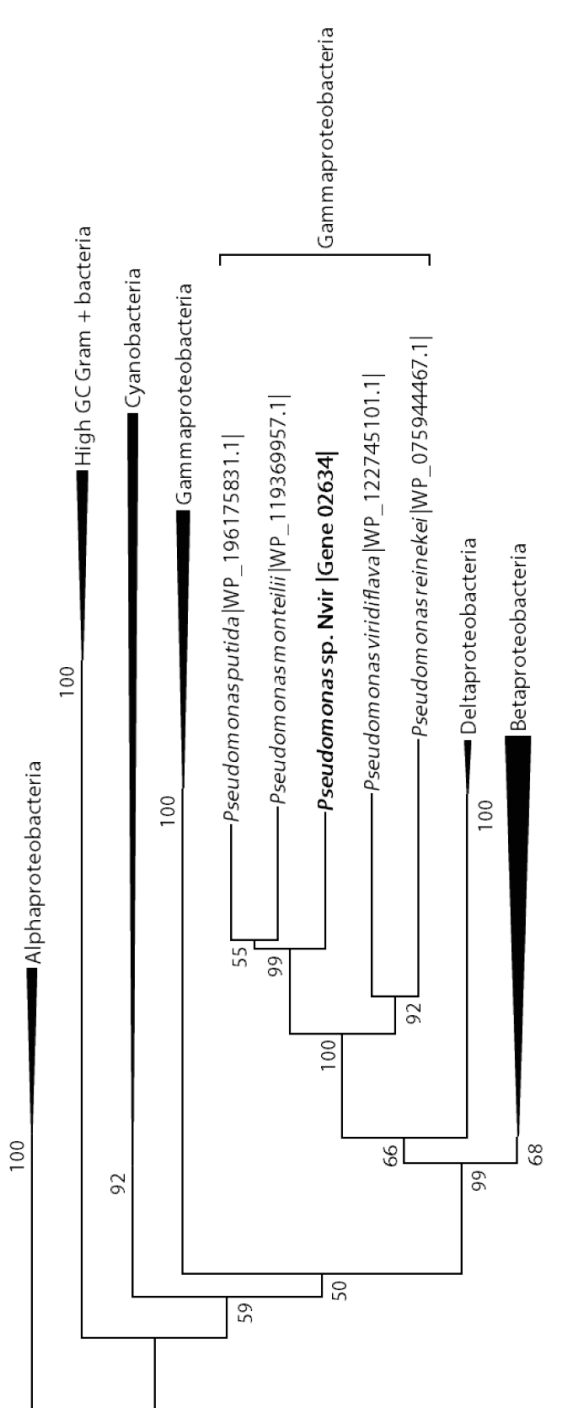


Figure 7.7. Neighbor-joining tree of *pnmR* homologs found in bacteria. Amino acid sequences were aligned with MUSCLE (Edgar, 2004) and phylogenetic trees were calculated with MEGA7 (Kumar et al., 2016) by using 500 bootstrap replicates. The values on the branches are the percentages of replicate trees in which the associated genes clustered together in the bootstrap test for values of ≥50. Branches are drawn to scale and branch lengths are in the same units as the evolutionary distances used to infer the phylogenetic tree. An unresolved phylogenetic tree is shown in Supplementary Figure 6.

Discussion

NPA and its glucose esters are widely distributed naturally occurring nitroaliphatic toxins. They were found to be produced by many leguminous plants, including over 450 different species and varieties of fungi (Awapak et al., 2021; Wei et al., 1994). To avert the toxic effect of NPA, nitrotoxin-producing plants and fungi contain enzymes that convert toxic nitroglycosides to non-toxic forms (Hipkin et al., 1999). On the account of the far-reaching appearance of NPA in nature, many animals, in particular cattle, annelids and insects counteract the adverse effects on nitrotoxins by harbouring gut-dwelling microorganism degrading NPA. The microbiota of NPA-exposed animals thereby protects their host from intoxication. So far, the ability to biodegrade nitrotoxins had been shown to be carried out via different enzymatic routes.

In the rumen of cattle, NPA glucose conjugates are rapidly hydrolysed liberating free NPA, which subsequently is either further metabolized by the ruminal microbiota to β-alanine and aminopropanol or absorbed into the circulatory system (Anderson et al., 2005). Along, the bacterial isolate from the gut of the earthworm *Eisenia fetida* (Opisthopora: Lumbricidae) was found to grow exclusively on NPA as the sole carbon, nitrogen and energy source (Saha et al., 2019). Contrarily, yet only hypothesised, a partial microbial role in NPA degradation was suggested in grasshoppers (*Melanoplus bivittatus* and *M. sanguinipes*; Orthoptera: Acrididae), where NPA detoxification occurs by the conjugation of the NPA with amino acids and subsequent excretion of NPA-amides by the insect (Majak et al., 1998). Nonetheless, bacteria isolated from other ecological niches were able to degrade NPA too. Similarly to the earthworm isolate, bacteria obtained from soil and water samples grew aerobically on NPA as the sole carbon, nitrogen, and energy source. As a result of NPA degradation, 3OP, nitrite, nitrate, and hydrogen peroxide were formed (Nishino et al., 2010).

In this study, we investigated the biodegradation pathway of NPA of a *Pseudomonas* sp. Nvir isolated from the gut of the pest insect *N. viridula*. The results indicate that both nitrate and nitrite were intermediary products which were transformed into ammonium and subsequently used as building blocks for the biosynthesis of amino acids. The isotopologue tracer experiment with ^{15}N-NPA showed the enrichment of ^{15}N-glutamine and ^{15}N-glutamate over time which indicates that NPA serves as a nitrogen source. Furthermore, CO_2 was released from the carboxylic acid group of NPA, yielding a two-carbon

by-product, which was further utilized in the central carbon metabolism of *Pseudomonas* sp. Nvir through the TCA cycle. In addition, the transcriptome analysis suggested that one of the transformation products of 3OP was β-alanine indicating that the NPA metabolism by *Pseudomonas* sp. Nvir was somewhat similar to that of rumen bacteria where accumulation of β-alanine was observed during NPA degradation (Anderson et al., 1993).

The biochemical fate of NPA in eukaryotes and bacteria has been extensively studied and the substantial differences within the NPA degradation pathways are now apparent. Yet, there still seems to be knowledge gaps in the characterization of the genes and enzymes involved in the degradation pathway. Currently, many identified enzymes involved in the NPA degradation are orphan enzymes (Lespinet & Labedan, 2006). The first crucial step in the degradation however seem to relay on flavoproteins which have oxidoreductive activity. Nitrotoxin-synthesizing plant and fungi produce various enzymes such as NPA oxidase (NPAO), P3NO, NMO, and nitroalkane oxidase (NAO) which transform NPA and its conjugate base to numerous intermediates (Anderson et al., 2000; Gorlatova et al., 1998; Hipkin et al., 1999; Kido et al., 1978; Kido et al., 1976; Nishino et al., 2010; Vercammen et al., 2015). Likewise, an NPA-degrading *Pradoshia eiseniae* gen. nov., sp. nov. isolated from the earthworm's gut encoded two homologous NMOs in the genome. Interestingly, *Pseudomonas* sp. JS189 harbored P3N monooxygenase encoded by *pno*A whereas in *P. aeruginosa* PAO1 NPA-degradation was catalyzed by NMO encoded by *nmo*A. This demonstrates that even bacteria within the same genus may produce different NPA-degrading enzymes. In this study, we observed that the previously unidentified gene *pnm*R was upregulated in *Pseudomonas* sp. Nvir under NPA plentiful conditions. Based on its phylogeny and metabolomic analysis, we hypothesised that the corresponding PNMR of *Pseudomonas* sp. Nvir harbours hence a similar catalytic activity as the only distantly related bacterial P3N monooxygenase and is a key enzyme in NPA degradation. It is conceivable that the enzymes similarly act upon NPA degradation as they both release nitrate and nitrite as the intermediate degradation products. Interestingly, NMO and NAO release no nitrate while degrading NPA (Nishino et al., 2010). Ultimately, to reveal whether PNMR is indeed a nitronate monooxygenase, further isolation of the enzyme and its biochemical characterization will be required.

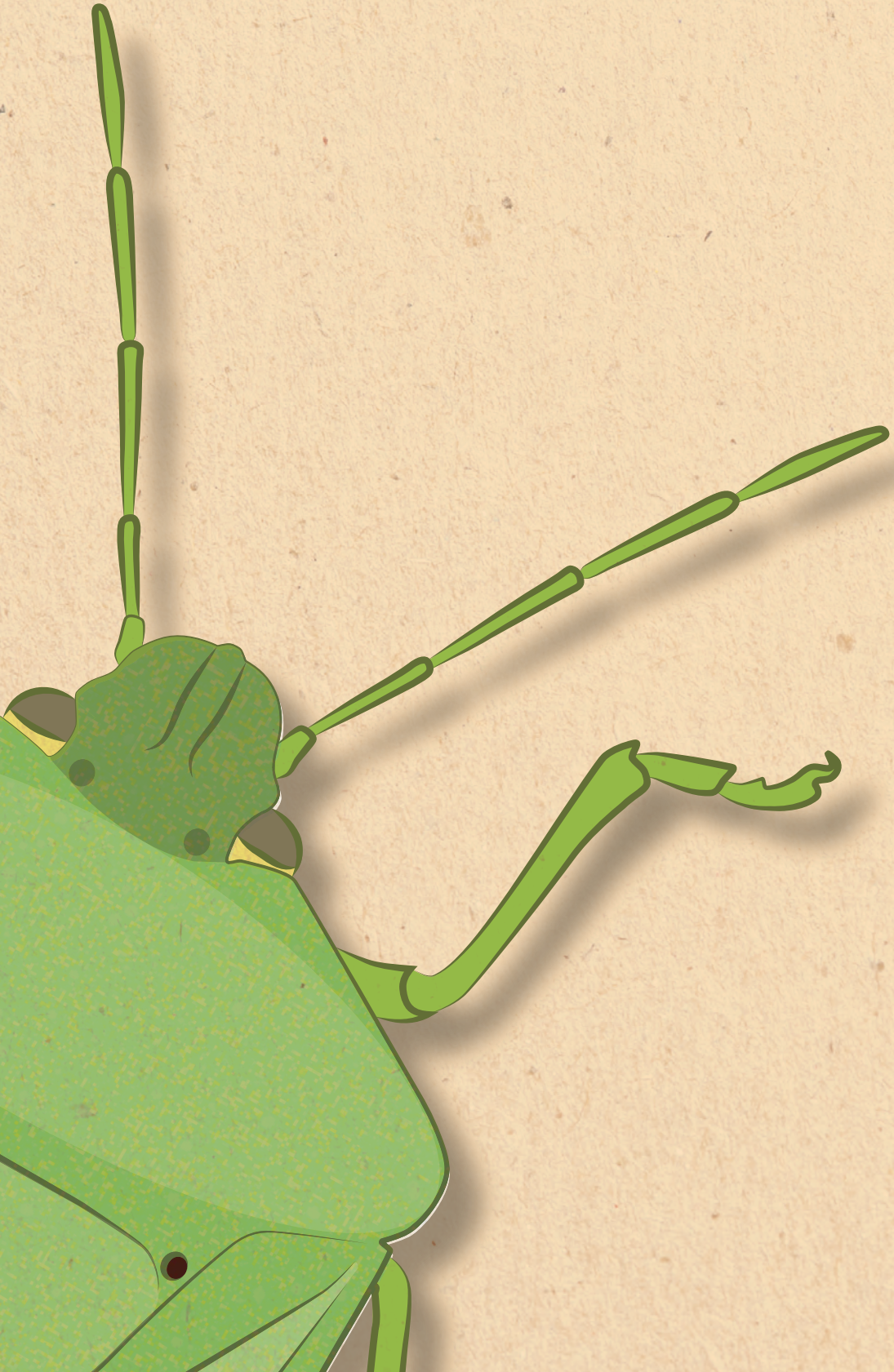
The functional characterization of the enzymes involved in the NPA degradation may provide insights into the bioconversion of NPA-contaminated products and could give rise to multiple applications in industry. As reported by Torres-

Guzman *et al.* nitrotoxin-degrading enzymes could be applied in biotechnology (Torres-Guzman et al., 2021). Nitronate monooxygenase are broadly distributed enzymes that have diverse catalytic efficiencies and preferred substrates which allows them to be used as pharmaceutical agents, biocatalysts and in bioremediation. Therefore, the characterization of PNMR found in *Pseudomonas* sp. Nvir might be a new mean in reducing the negative impact of NPA in many important industrial sectors.

To conclude, the results of this study revealed that the *pnm*R gene could encode an enzyme for NPA degradation. By using an array of complementary techniques, we shed light on the metabolic potential of *Pseudomonas* sp. Nvir and established its degradation pathway. With this study, we, therefore, provide insights into the microbial degradation and tolerance mechanisms towards toxic nitroglycosides which suggests possible applications of bacteria from understudied ecosystems in the industry.

So far, degrading bacteria were found to be acquired by insects to perform symbiont-mediated detoxification, through which insects gain protection against toxic plant metabolites and insecticides (van den Bosch & Welte, 2017). Currently, the role of the *Pseudomonas* sp. Nvir in the Southern green shield bug remains unclear, and from our research project, it is not possible to deduce whether the isolate is a mutualistic symbiont in the insect's gut microbiome or whether it is a transient environmental bacterium. It is however tempting to speculate that as other gut-dwelling microorganism, the NPA-degrading *Pseudomonas* sp. Nvir might serve its host by protecting it against the toxic influence of nitroglycosides.

Acknowledgements
We thank Jim Spain for providing us with an NPA-degrading bacterial isolate that was used in a preliminary study on the NPA degradation mechanism, and Huub Op den Camp for help with the phylogenetic trees. The work was funded by the Nederlandse Organisatie voor Wetenschappelijk Onderzoek through Gravitation Grant SIAM 024.002.002.



Chapter 8

Discussion and Outlook

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Nezara viridula is a highly invasive herbivorous insect with a broad dietary spectrum covering over 30 plant families, with preference extending to such crops as tomatoes (Solanaceae), cabbage (Brassicaceae) and legumes (Fabaceae) (McPherson & McPherson, 2000; Todd, 1989). It is globally spread which results in decreased crop yield and substantial financial losses in the agricultural sector, making *N. viridula* a major pest insect (Jones, 1988). Similar to other shield bugs, *N. viridula* is a piercing and sucking insect that feeds on the vascular fluids of fruits, leaves, and pods (Depieri & Panizzi, 2011; Velikova et al., 2010). The insect injects saliva, containing enzymes like proteases and nucleases, into the plant to initiate digestion (Lomate & Bonning, 2016). The puncture of plant tissue and transmission of saliva triggers plant defence mechanisms, including the biosynthesis of toxic secondary plant metabolites and protease inhibitors, and the induction of tri-trophic systems to deter insects (Labandeira, 2007; Smith & Clement, 2012). In response to these natural defences, shield bugs like *N. viridula* have evolved strategies to overcome plant resistance, often by forming symbiotic relationships with microbes. A recent study has revealed that the microbiota associated with *N. viridula* plays a crucial role in detoxifying soybean isoflavonoids and deactivating protease inhibitors (Medina et al., 2018). Studies like these are very scarce, however, broadening our understanding of pest adaptation mechanisms could contribute to developing effective strategies for pest management in agriculture.

In the past years, shield bugs' microbiota has been extensively studied, yet many fundamental questions await investigation. Previous research has confirmed that shield bugs host their obligate *Pantoea* symbiont in midgut crypts, however the role of other symbionts in various gut regions and different organs remains largely unexplored to date (Duron & Noel, 2016; Karamipour et al., 2016; Kashkouli et al., 2021; Prado et al., 2009; Ragsdale et al., 1979; Tada et al., 2011; Walterson & Stavrinides, 2015). In this PhD thesis, we addressed these knowledge gaps and aimed at understanding the relationship between *N. viridula* and its associated microbes. Chapters 3 to 7 explored the insect-microbe symbiosis, unravelling the role of *N. viridula*'s microbiota as illustrated in Figure 8.1. To this end, in **Chapter 3**, we first characterized the core microbiota of *N. viridula*, determined the symbiont transmission routes and demonstrated that symbionts play a crucial role in detoxification and repression of plant defences. Our research described in **Chapter 4** demonstrated the obligate nature of the symbiont *Sodalis* and its predominant localization in salivary glands, anterior regions of the midgut and testes. In **Chapter 5**, we characterized a novel *Commensalibacter* sp. Nvir symbiont and indicated that

the species differs from other bacteria within the *Commensalibacter* genus by having the metabolic potential to degrade the solanaceous toxins α-solanine and α-chaconine. In **Chapter 6**, we unveiled that diet had a substantial impact on the *N. viridula* gut microbiota community composition and determined microbe-microbe interactions between members of the core and transient microbial community. Our findings showed that most *N. viridula*-associated microbes could degrade 3-nitropropionic acid and suggested that the toxin could inhibit the transport and biosynthesis of amino acids in *Pantoea*. Lastly, in **Chapter 7**, we characterized *Pseudomonas* sp. Nvir isolated from the gut of *N. viridula*, reconstructed the 3-nitropropionic acid breakdown pathway, and discovered a novel *pnm*R gene that is likely involved in the first step of the degradation pathway. In the following sections, our findings are used to discuss these topics, concluding by highlighting future research perspectives in the field of insect-microbe symbiosis.

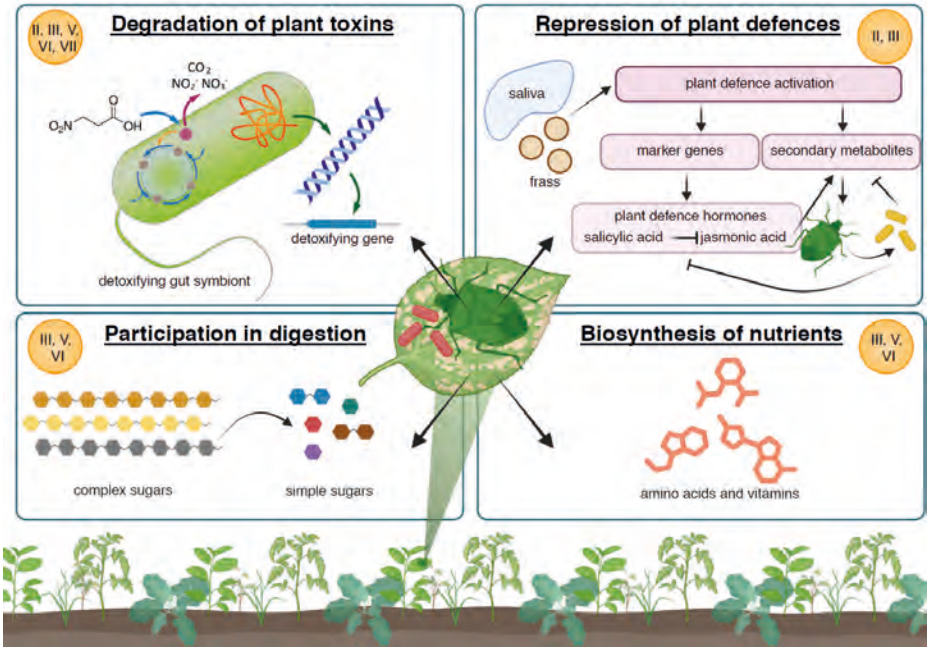


Figure 8.1. The roles of *Nezara viridula* microbiota. *N. viridula* harbours symbiotic bacteria which benefit their host. Based on the findings of this PhD thesis, we determined that *N. viridula*-associated microbiota participates in the degradation of toxic secondary plant metabolites, altering plant defences, degradation of complex carbohydrates to simple molecules and biosynthesis of essential amino acids and vitamins. Roman numerals indicate Chapters where the specific role has been investigated or discussed. Created with BioRender.com.

N. viridula's microbiota is capable of overcoming plant defences

To deter insects, plants deploy chemical defences that are induced upon recognition of potential threats. In case of piercing and sucking insects such as *N. viridula*, puncturing of plant tissue triggers the activation of attacker-specific defences, resulting in the production of toxic secondary plant metabolites (Chauvin et al., 2013). Insects have coevolved to cope with plant resistance and pesticides by acquiring symbiotic microorganisms which aid in breaking down toxins through detoxifying symbiosis (Almeida et al., 2017; Ceja-Navarro et al., 2015; Coolen et al., 2022; Frago et al., 2012; Medina et al., 2018; van den Bosch & Welte, 2017; Welte, de Graaf, et al., 2016; Welte, Rosengarten, et al., 2016). For example, a previous study showed that the bean bug's gut is colonized by symbiotic bacteria which degrade the insecticide fenitrothion (Sato et al., 2021). In this symbiosis, fenitrothion is converted into a non-toxic compound by bacteria and excreted by the host insect. Interestingly, the authors determined that the insecticide-degrading enzyme is horizontally acquired, implying that other detoxifying genes could be easily spread in the environment, affecting insect resistance to toxic compounds.

The participation of *N. viridula*'s microbiota in the detoxification of soybean isoflavonoids was recently determined, but the exact process mediated by symbionts remained unclear (Medina et al., 2018). Thus, to unveil how *N. viridula*'s microbiota is involved in detoxification and overcoming plant defences, we first characterized the insect microbiota associated with the gut, salivary glands and eggs (**Chapter 3**). Our findings revealed that the *N. viridula* microbiota primarily comprised of highly abundant *Pantoea*, *Sodalis*, *Serratia*, and *Commensalibacter*, collectively termed the core microbiota. In contrast, Medina et al. (2018) identified *Yokenella*, *Pantoea* and *Enterococcus* as the main constitutes of the core microbiota which confirmed observations from a previous study, that microbiota of the same insect species from different geographical populations might differ (Hosokawa et al., 2016). Furthermore, we observed less prevalent microbes including *Klebsiella*, *Pseudomonas* and *Cutibacterium*, which were likely part of the transient microbial population. Through the analyses of the egg microbiome and the microbiota profile across developmental stages, we determined that *Pantoea* and *Sodalis* were vertically obtained whereas other *N. viridula*-associated microbes were likely horizontally transmitted from the environment (**Chapter 6**; (Geerinck et al., 2022; Perlmutter & Bordenstein, 2020)). The microbiome of shield bugs was found to be acquired

by the transmission of the maternal microbiota to the offspring via egg smearing on the egg surface (Calizotti & Panizzi, 2014; Fukatsu & Hosokawa, 2002; Kikuchi et al., 2007; Prado et al., 2006; Tada et al., 2011). Therefore, surface sterilization of the eggs' outer layer, in the future, offers insights into the potential internal transmission of symbionts (Binetruy et al., 2019). Furthermore, performing experiments, where insects are reared in aseptic conditions and infected with specific microbes with subsequent assessment of microbe presence in the offspring could significantly expand our understanding of symbiont transmission routes (Masson & Lemaitre, 2020).

In addition, our data indicated the obligate nature of *Pantoea*, which is supported by several authors who have reported that the elimination of *Pantoea* leads to increased mortality (Bistolas et al., 2014; Prado & Almeida, 2009). *Pantoea* symbionts are often associated with pentatomid shield bugs like *N. viridula*, and the stable relationship between *Pantoea* and their host appears to date back to the origins of genus subdivisions within the insect group (Duron & Noel, 2016; Hosokawa et al., 2016; Karamipour et al., 2016; Kashkouli et al., 2021; Prado et al., 2009; Ragsdale et al., 1979; Tada et al., 2011; Walterson & Stavrinides, 2015). Typically, only one *Pantoea* symbiont is found per host species. However, our metagenomic analysis revealed the concurrent presence of multiple *Pantoea* species. Along with that, we were capable of isolating and cultivating one of those *Pantoea* symbionts, whose genome was described in detail in **Chapter 6**. The metagenome of *Pantoea* described here was highly reduced which suggested that the symbiont depend on their hosts, making them unable to sustain growth independently (Lo et al., 2016). Interestingly, the genome of *Pantoea* isolate differed in size from the *Pantoea* metagenome characterized in **Chapter 3** but demonstrated traits of genome instability typical for endosymbiont lineages (Manzano-Marin & Latorre, 2016; Manzano-Marin et al., 2017). This observation implied ongoing speciation from its free-living ancestor to an obligate symbiont, explaining the ability of *Pantoea* isolate to survive independently of the host. A previously conducted study on *Pantoea* diversity among shield bugs indicated that *Pantoea* symbionts may be lost and replaced by others that were recently acquired from the environment or another insect species (Duron & Noel, 2016). Therefore, we hypothesize that a similar phenomenon might have occurred in *N. viridula*. Nonetheless, a follow-up evolutionary analysis involving both *N. viridula*-associated *Pantoea* symbionts, together with the experimental introduction of the defined microbiota to the insect in a similar manner shown by Wierz et al. (2021), is required to validate this theory.

While feeding, insects transmit microbes and plant-degrading enzymes through saliva, facilitating the digestion of plant material and supporting nutrient uptake (Bonaventure et al., 2011; Lomate & Bonning, 2016). Insects reared under laboratory conditions were suggested to lack saliva microbiota, as stress-induced salivation did not yield any microbial components (Giacometti et al., 2020). In our research, we observed dense colonization of salivary glands by *Sodalis* and showed that it was transmitted by *N. viridula* during feeding, in contrast to earlier findings. Moreover, the metagenomic analysis of *Sodalis* revealed that the strain had the metabolic potential to degrade starch and D-galacturonate and thus could participate in pre-digestion, benefitting the host. We found that *Sodalis* encodes a type III secretion system, which could allow cellular invasion, modulation and repression of plant defences through the secretion of effector proteins (Ceulemans et al., 2021). Eventually, this could potentially reduce the synthesis of insect-deterring compounds and toxic metabolites (Coolen et al., 2022; McHugh et al., 2019). In case of the Colorado potato beetle, it was shown that transmission of microbiota during feeding represses plant jasmonic acid defences that are directed against insects. This repression was caused by microbial salicylic acid induction that repressed the plant's jasmonic acid-defensive pathway via negative crosstalk (Caarls et al., 2015; der Does et al., 2013; Robert-Seilaniantz et al., 2011). Also, whitefly *Bemisia tabaci* was shown to repress plant defences in a similar manner (Su et al., 2015). To assess whether *N. viridula*'s microbiota is capable of altering plant defences, we performed a plant assay experiment and determined that *Sodalis* was the only microbe that significantly repressed the aliphatic glucosinolate pathway, involved in the production of toxic secondary metabolites. This finding was in line with the metagenomic predictions of the type III secretion system. Altogether our observations, combined with *Sodalis*' metabolic potential, its high abundance in salivary glands, and its vertical transmission route, strongly suggest an obligate nature of the *Sodalis* symbiont.

Although we were able to determine that *Sodalis* is a key player in repressing plant defences, the rapid plant defence signalling system results in the production of antifeeding compounds and toxic secondary metabolites that predominantly target the insect's gut system (Coolen et al., 2022; Mao et al., 2011; Mason et al., 2019; Schlaeppi et al., 2008). Microbes, many of which are symbiotically associated with insects, are well-known to degrade toxic plant metabolites (**Chapter 2**). Upon the recent discovery of the ability of *N. viridula* microbiota to detoxify soybean metabolites, we hypothesized that our laboratory-reared *N. viridula* might harbour symbiotic gut bacteria which

could facilitate the degradation of other toxins. Indeed, we were able to isolate several 3-nitropropionic acid (NPA)-degrading bacteria (**Chapter 3, 6, 7**) and determined that *N. viridula* core- and transient-associated microbes could rapidly degrade the toxin *in vitro*, potentially protecting the host against the toxin. Moreover, metagenomic analysis of the *Commensalibacter* symbiont revealed that the strain had α-rhamnosidase, β-galactosidase and β-glucosidase genes which encode enzymes essential for the breakdown of metabolites with insecticidal properties – α-solanine and α-chaconine (Hennessy et al., 2020). W. Wang et al. (2022) also found that *Glutamicibacter halophytocola* mediated the resistance of potato tuber moth to α-solanine and α-chaconine and harboured identical genes, suggesting potential horizontal transfer of detoxifying genes among microbes associated with insects. Feeding on toxic plants exerts selective pressure on insects and their gut microbiota, indicating that such microbial adaptations as horizontal gene transfer, beneficial to the host insect are likely to occur (Vilanova et al., 2016). Eventually, this selective pressure could increase insects' host range, including the spread of *N. viridula* to other plant species.

In conclusion, we have characterized the microbial community of *N. viridula* and demonstrated that gut- and salivary glands-associated microbes likely play crucial roles in overcoming plant defences. However, to fully verify that the *N. viridula* microbiota mediates detoxification and plant defence repression, eliminating the *N. viridula* microbiome is required. Nonetheless, to this day, eliminating all microbes remains challenging since *N. viridula* requires its microbiota for viability and development (**Chapter 4**).

Sodalis functions as essential obligate symbiont

Many symbionts are essential for the viability of their shield bug hosts (Douglas, 2011). Experimental removal of the external microbiome from egg masses of *N. viridula,* for example*,* disrupted nymphal infection with symbionts and severely increased nymphal mortality (Tada et al., 2011). Next to *Pantoea* located in the M4 crypts (Bistolas et al., 2014; Hosokawa et al., 2016; Kikuchi, Hosokawa, et al., 2012; Taylor et al., 2014), it has been suggested that shield bugs might harbour *Sodalis* as a core symbiont in anterior gut compartments, where it might fulfil a role in nutrient provisioning for the host (Fourie et al., 2023; Hosokawa et al., 2015). Nothing is currently known about the presence and abundance of *N. viridula*-associated bacteria in organs other than the gut

and this knowledge could be crucial to understand symbiont transmission to the offspring. Our characterization of *N. viridula* microbiota (**Chapter 3**) indicated the high abundance of *Sodalis* in salivary glands and suggested an obligate nature of the symbiont. Therefore, to determine the status of the symbiont, we delved into investigating *N. viridula*-associated *Sodalis* sp. Nvir including analysis of its phylogeny (**Chapter 4**).

Upon the recent discovery of an internal egg microbiota (Geerinck et al., 2022), we questioned how the removal of symbionts from the egg surface impacts *N. viridula* survival rate and acquisition of symbionts. We observed that surface sterilization negatively affected insect viability and caused retarded growth, in line with the findings of Prado et al. (2009) and Tada et al. (2011) who found that surface sterilization drastically decreased *N. viridula* vitality and development. In addition, our findings demonstrated that *Sodalis* abundance decreased in the sterilized group but, along with *Pantoea*, still dominated the gut bacterial community in adult insects. Similarly, Prado et al. (2006) reported that up to 60% of *N. viridula* nymphs hatched from surface-sterilized eggs acquired symbionts. This, along with the detection of *Sodalis* and *Pantoea* with diagnostic PCR in sterilized eggs, suggested a probable origin of symbionts from the centre of the egg. Insects were previously shown to internally transmit endosymbionts via eggs; for instance *Candidatus* Stammera capleta in tortoise leaf beetles or *Buchnera* in pea aphids (Koga et al., 2012; Salem et al., 2017; Salem et al., 2020). Altogether, our data suggested the ability of *N. viridula* to retain *Sodalis* and *Pantoea* in the event of external egg microbiome disturbance and maintain vertical transmission of microbes via internal storage of symbionts in eggs.

Furthermore, we showed that *Sodalis* predominantly colonized salivary glands, anterior regions of the midgut and testes. Surprisingly, even though other authors reported the presence of *Sodalis* symbiont in the ovaries of a jewel shield bug, we were not able to visually locate any insect-associated microbes in *N. viridula's* ovaries (Kaiwa et al., 2010). We hypothesize that the spatial organization of the symbiont in testes was likely linked to its role in paternal transmission, which was previously reported to occur in insects. Tsetse flies were shown to maternally and paternally transmit obligate intracellular *Wolbachia* symbiont, illustrated by the detection of microbes in ovaries and testes (Doudoumis et al., 2012). Likewise, *Sodalis glossinidius* was shown to be transmitted from males to females during mating (De Vooght et al., 2015) and Watanabe et al. (2014) discovered that bacterial symbiont *Rickettsia* is vertically transmitted via sperm in leafhopper *Nephotettix cincticeps*. Also,

paternal transmission of bacteria was observed in *Anopheles stephensi* mosquitos (Damiani et al., 2008). However, there has been limited focus on the male reproductive organs within the Pentatomidae family. Although speculative, it is conceivable that paternal transmission of symbionts can occur in *N. viridula* via the internal egg microbiome. Confirmation of this hypothesis requires further research.

Additionally, we conducted a comparative genomic analysis of various *Sodalis* species, revealing that *Sodalis* sp. Nvir shares characteristics with the free-living *S. praecaptivus* and the obligate host-associated *S. pierantonii* strain SOPE. Recent studies have identified a lineage within the *Sodalis* genus that transitions into vertically transmitted endosymbionts marked by massive genome reorganization of reduction (Clayton et al., 2012; Oakeson et al., 2014; Toh et al., 2006). Our Nvir strain displayed a large number of plasmids which suggested genome instability and adaptation towards a symbiotic lifestyle (Campbell et al., 2017; Van Leuven et al., 2014). In summary, our findings point to the divergent nature of *Sodalis* sp. Nvir and show the strain's evolutionary journey from a non-host-associated ancestor to an obligate symbiont of *N. viridula*.

Commensalibacter sp. Nvir adapted to a symbiotic lifestyle

The gut microbiota of shield bugs and other true bugs (Heteroptera) is mainly located in the midgut and predominantly consists of Gammaproteobacteria (*Buchnera, Pantoea*, *Ca.* Ishikawaella capsulata) and Betaproteobacteria (*Burkholderia*) (Kikuchi, Hosokawa, et al., 2012; Medina et al., 2018; Ozsahin et al., 2014; Shan et al., 2021). Our research revealed that besides Gammaproteobacteria, *N. viridula* harbours Alphaproteobacteria and particularly a novel strain *Commensalibacter* sp. Nvir in the gut (**Chapter 5**). *Commensalibacter* belongs to the *Acetobacteraceae* family, commonly referred to as acetic acid bacteria (AAB). AAB are ubiquitous, obligate aerobes, widely spread in sugar-rich environments such as fruits, flowers and nectar. For this reason, they often form symbiotic relationships with plant-sap-feeding insects (Bartowsky & Henschke, 2008; Favia et al., 2007; Roh et al., 2008; Ryu et al., 2008; Servin-Garciduenas et al., 2014; Siozios et al., 2019). A recent study unveiled that AAB have functional traits for adaptation to symbiotic life which explains their prevalence in insects (Chouaia et al., 2014). *Commensalibacter* is a core member of the gut microbial community of honey bees, bumble bees,

monarch butterflies, fruit flies, and hornets and has been associated with host health. Studies investigating the immune response of fruit flies revealed that the host regulates the composition of gut microbes by favouring *C. intestini,* which in return acts opportunistically against pathogenic *Gluconobacter* (Botero et al., 2023; Martinez et al., 2019; Roh et al., 2008; Ryu et al., 2008). Nevertheless, *Commensalibacter* is still poorly studied, and little is known about its origin, functional diversity, and role in insect life.

Despite efforts, we were unable to isolate the strain from the gut of *N. viridula.* To unveil *Commensalibacter* metabolic potential and adaptations toward symbiotic lifestyle, we performed a comparative genomic analysis on *Commensalibacter* genomes associated with insects (**Chapter 5**). We collected five publicly available genomes of *Commensalibacter* from honey bees, monarch butterflies, and common fruit flies, and compared them to the metagenome of our *Commensalibacter* sp. Nvir. We hypothesized that due to the low abundance of *Commensalibacter* sp. Nvir in the gut, the microbe likely functions as a facultative symbiont. This aligns with previous reports that indicated no negative effects on the host after antibiotic removal of AAB from mosquitos (Favia et al., 2007). Our investigation revealed that *Commensalibacter* possessed a reduced genome and a relatively low GC content suggesting adaptations to symbiotic lifestyle as a result of small population size, decreased recombination efficacy and constant nutrient environment (Van Leuven & McCutcheon, 2012). Furthermore, the phylogenomic analysis positioned *N. viridula*'s symbiont as a novel strain within the *Commensalibacter* genus. Our study also identified *Commensalibacter* sp. Nvir contained the largest amount of unique protein-coding sequence clusters and the most extensive carbohydrate-degrading potential. These findings imply species-dependent adaptations to the host's diet. Previous research has established that diet is the driving force in the evolution of symbiosis and gene acquisition might reflect the close relationship between the *Commensalibacter* sp. Nvir and *N. viridula* (Amato et al., 2019; Martino et al., 2018).

As a member of the insect gut community, *Commensalibacter* holds the potential to fulfil host nutritional requirements by supplementing amino acids and vitamins (Jing et al., 2020). Our research showed that *Commensalibacter* is capable of the biosynthesis of seven essential amino acids and most B vitamins. However, it is noteworthy that *Commensalibacter* sp. Nvir lacked the capability to produce proline, leucine and methionine and synthesizes threonine differently compared

to other strains. These observations suggest that *Commensalibacter* sp. Nvir is likely undergoing a reorganization of the genome.

Moreover, our investigation unveiled genomic adaptations in *Commensalibacter* as indicative of a symbiotic lifestyle. These adaptations include the presence of genes involved in modulating host-microbe signalling and stress response to phosphate starvation, which is limited in the diet of plant-sap-feeding insects (Feehily & Karatzas, 2013; Hsieh & Wanner, 2010; Quillin et al., 2021; Siragusa et al., 2007; Wang et al., 2021). Besides this, bacterial survival in establishing a stable symbiotic relationship with the host often involves toxin-antitoxin systems (TAs) that are activated upon environmental stresses. Notably, we found that *Commensalibacter* sp. Nvir has the largest amount of TAs of all analysed genomes, reflecting potentially harsher environmental conditions within *N. viridula*'s gut compared to what other *Commensalibacter* strains experience. We hypothesise that these conditions might be the result of the toxins present in the diet of *N. viridula*, therefore we analysed the metabolic potential of *Commensalibacter* to degrade secondary plant metabolites. Our findings demonstrate that solely *Commensalibacter* sp. Nvir had the metabolic potential to fully degrade α-chaconine and α-solanine and facilitate detoxification in the insect's gut. Altogether, with this research, we broadened our understanding of *Commensalibacter*'s function in the insect gut and illustrated that *Commensalibacter* not only potentially provides nutrients and digests food but also has the potential to degrade toxic secondary plant metabolites. Future research focusing on the isolation of *Commensalibacter* sp. Nvir through techniques such as cell sorting or co-culturing could confirm the aforementioned benefits that *Commensalibacter* provides to the host.

Core and transient microbes facilitate the degradation of 3-nitropropionic acid

The insect microbiome consists of core and transient microbes which can contribute to the host's digestion, development, pathogen resistance, detoxification, and physiology (Douglas, 2015; Engel & Moran, 2013; Jing et al., 2020). Medina et al. (2018) recently showed that the core microbiota of *N. viridula* was involved in soybean isoflavonoids detoxification. However, the role of transient microbiota remained elusive. In our laboratory, *N. viridula* feeds on another leguminous plant, crown vetch, which produces NPA to deter insects (Al-Snafi, 2016) yet we have not observed any signs of intoxication in insects.

When ingested by mammals, NPA causes irreversible inhibition of succinate dehydrogenase in the TCA cycle, impairing the function of the electron transport chain and causing the accumulation of toxic nitrite ions that disrupt haemoglobin (Anderson et al., 2016; Francis et al., 2013). Microbial detoxification of NPA has been shown in soil microbes and gut-associated bacteria isolated from rumen and insects (Anderson et al., 1993; Majak et al., 1998; Nishino et al., 2010). NPA serves bacteria as carbon, nitrogen and energy sources and hence maintaining NPA-degrading capabilities is favourable (Nishino et al., 2010). Moreover, the widespread occurrence of microbes with NPA-degrading abilities indicates the horizontal transfer of detoxifying genes, which eventually could lead to the broad distribution of NPA-degrading capabilities among insect-associated microbes (Lee et al., 2022).

Detoxification of NPA is carried out by either nitronate monooxygenase (NMO), encoded by *nmo*A or *pnm*R, or by propionate-3-nitronate monooxygenase (P3NO) encoded by *pno*A. Despite structural differences, these three enzymes all transform NPA to the non-toxic intermediate 3-oxopropanoate with subsequent release of nitrate and nitrite (**Chapter 7**). Given the apparent resistance of our *N. viridula* to NPA-rich crown vetch, we explored the potential involvement of core and transient gut-associated microbiota in the degradation of NPA (**Chapter 6**). Four core (*Pantoea*, *Sodalis*, two *Serratia*) and four transient microbes (*Pseudomonas*, *Klebsiella*, two *Bacillus*) associated with *N. viridula* were isolated and sequenced, and genomic analysis revealed that seven strains had the metabolic potential to degrade NPA. Subsequent culturing of these bacteria with NPA indicated that *Pantoea*, *Serratia* spp., *Pseudomonas* and *Bacillus* spp. were capable of NPA degradation, albeit at different rates. Surprisingly, while our genomic analysis indicated that *Klebsiella* harboured *pno*A and therefore had the potential to metabolize NPA, NPA degradation was not observed. Similarly, in **Chapter 7**, we showed that although *Pseudomonas* sp. Nvir harboured *pno*A, but the gene was not expressed, and instead *pnm*R was used to degrade NPA. Recent studies reported that the expression of *nmo*A and *pno*A is regulated by a LysR regulator, and ΔLysR mutants could not degrade 3-nitropropionic acid (Nishino et al., 2010; Vercammen et al., 2015), suggesting that, unlike other NPA-degrading microbes, *Klebsiella* and *Pseudomonas* might have lacked LysR regulator. Furthermore, despite the lack of NPA degradation by *Klebsiella*, the strain showed the highest resistance against the toxin, suggesting that besides detoxification, bacteria have evolved other mechanisms to defend themselves against toxins. They often rely on efflux pumps that actively transport toxins out of the cell or modify toxin structures

within their cells (Li & Nikaido, 2009; Walsh, 2000). In addition, bacteria living in communities could also use quorum sensing to communicate with each other and initiate responses against toxins (Borlee et al., 2008; Mondal et al., 2023). This could be particularly important in such systems as insects' gut where toxin-degrading bacteria can protect not only the host but also other microbial members, thus preventing a collapse of the microbial community. It would be interesting to determine whether microbes found in *N. viridula* gut communicate with one another to protect non-degrading members from the adverse effects of NPA.

The influence of NPA on the physiology of mammals is well-documented (Anderson et al., 2005; Kadir et al., 2022; Ludolph et al., 1991) but little is known regarding its impact on bacteria. We analysed the transcriptome and metabolome of the NPA-degrading symbiont *Pantoea* and determined that NPA triggered a stress response which resulted in altered transport and biosynthesis of amino acids. Particularly L-leucine biosynthesis seemed to be inhibited by NPA since exposure caused intracellular accumulation of L-leucine biosynthesis intermediates such as 2-isopropylmalate. A recent study on the fungus *Aspergillus fumigatus* confirmed that the inhibition of LeuC and LeuA leucine biosynthesis enzymes led to the cellular accumulation of 2-isopropylmalate, resulting in leucine auxotrophy (Orasch et al., 2019). While our findings suggested that NPA might impair bacterial amino acid supplementation to the host and as a result fail to support insect nutritional requirements, several questions remained unanswered. A number of studies indicated that the accumulation of intermediate products could be toxic to cells (Amarnath et al., 2023; Heipieper & Martínez, 2010; Panigrahy et al., 2022; Richie et al., 2016), however little is known about the potential toxicity of L-leucine intermediates. To ascertain whether the accumulation of these intermediate metabolites triggers toxicity in bacteria, future investigations could involve culturing various bacterial species in an NPA-free medium supplemented with increasing concentrations of 2-isopropylmalate. Quantifying L-leucine concentrations in these cultures, coupled with the determination of cell viability with colony-forming unit assays, could provide valuable insights into the effect of 2-isopropylmalate accumulation on L-leucine biosynthesis and deliver fundamental knowledge regarding the influence of NPA on bacterial physiology.

In **Chapter 6**, we investigated whether *N. viridula*-associated microbes were involved in the detoxification of NPA, eventually protecting the host. However, Beran et al. (2019) have recently described that insects have coevolved with

their host plants, and themselves harbour a wealth of adaptations that allow them to feed on toxic plants. These resistance mechanisms include target-site adaptations, inactivation via alkalization, rapid excretion, sequestration, degradation, and detoxification via cytochrome P450 monooxygenases, glutathione transferases and carboxylesterases (Li et al., 2007; Winde & Wittstock, 2011). Even though insects themselves adapted to cope with toxic compounds, they often acquire symbiotic microorganisms that aid in breaking down toxic plant metabolites (Almeida et al., 2017; Ceja-Navarro et al., 2015; Frago et al., 2012; Itoh et al., 2018; van den Bosch & Welte, 2017). Our data suggests that this has likely also occurred in *N. viridula* since we were able to isolate several NPA-degrading bacteria. Despite that, the capability of the insect to detoxify NPA remains to be further explored. The generation of gnotobiotic insects by feeding adult individuals with antibiotics as described by W. Wang et al. (2022) could allow for answering the nurturing question if insects fully rely on the collaboration with symbionts or whether symbionts only support the insects' resistance.

Core microbiota supports insect feeding strategy and antagonizes microbial invaders

Several factors determine insect microbiota, among which diet, environment and host taxonomy play crucial roles (Amato et al., 2019; Colman et al., 2012). Interestingly, the gut bacterial community composition of evolutionarily distinct insects is similar during feeding on the same diet; for instance, Huang et al. (2021) determined that gut microbes facilitate convergent evolution during the adaptation to the same diet in phylogenetically distinct species. Taken together, this suggests that microbes could contribute to the hosts' adaptations to changing environments (Itoh et al., 2018; Sato et al., 2021; van den Bosch & Welte, 2017).

A recent study showed that microbes that live in a consortium, such as insects' guts, might depend on each other by biosynthesizing and exchanging metabolites (Zhang et al., 2018). Thus, we questioned whether members of the *N. viridula* gut community interact with each other and whether community composition changes upon diet shift (**Chapter 6**). We unravelled microbial interactions between *N. viridula*'s gut microbiota members and demonstrated that in an artificial plant sap (PS) medium, bacteria formed a complex community network. A clear relationship was observed between *Sodalis* and

Pantoea, and *Serratia* strains, which had a strong mutualistic relationship and promoted each other's growth in the sugar-rich and nutrient-deficient PS medium. These findings suggested metabolite exchange via cross-feeding between bacteria and indicated that core microbes adapted to life in proximity (Blasche et al., 2021; Blasche et al., 2017; Goldford et al., 2018). While core microbiota thrived in the PS medium, the growth of bacteria associated with the transient microbiota was largely inhibited by either transient or core microbiota, implying a lack of adaptation to grow on phloem sap. As a result of the high concentration of sucrose in phloem sap (Broussard et al., 2023) transient bacteria might experience osmotic shock rendering their abilities to grow on insects' diet (Heppel, 1969). Consequently, our findings indicated the adaptive strategies and role of microbes in shaping the insect gut microbial community.

According to many studies, diet was determined as one of the most important factors in shaping the gut microbial community (Huang et al., 2021; Luo et al., 2021; Yun et al., 2014). Li et al. (2022) investigated the gut microbial biodiversity among true bug species and showed that carnivorous true bugs had substantially different gut microbiomes compared to herbivorous species. Likewise, gut microbial community analysis of the mirids bug *Adelphocoris suturalis* showed that the switch in diet affected the abundance and composition of insects' gut microbiome (Luo et al., 2021). Shield bugs feed on sugar-rich phloem sap and depend on their symbiotic microbial partners to fulfil their nutritional needs (Karamipour et al., 2016), however only a few studies explored the direct influence of diet on gut microbiota composition (Medina et al., 2022; Medina et al., 2018). Therefore, we investigated how diet influences the *N. viridula* gut microbiota, and determined that *Pantoea*, *Sodalis*, *Enterococcus*, *Serratia* and *Commensalibacter* dominated the gut while feeding on a multitude of plants. Switching from a polyphagous to a single-plant diet caused a shift in core microbial community composition which implied an overall stability of the gut microbiome in response to dietary shifts. Similar findings were reported by Medina et al. (2018) who observed the change in the relative abundance of core *Pantoea*, *Yokenella* and *Enterococcus*, during the transition of *N. viridula* between host plants, suggesting that stable core microbiota is important to the host.

Furthermore, when comparing the gut microbial composition of black mustard and black nightshade populations an evident shift in gut microbial relative abundance was noted. As previously shown in camellia weevils, this variance could potentially result from the production of distinct toxic secondary plant

metabolites directed against insects (Zhang et al., 2020). Black nightshade is known for the production of solanaceous toxins α-solanine and α-chaconine and the observed increase in *Sodalis* abundance could be linked with its potential capability to degrade toxins (Mohy-ud-Din et al., 2010). Hennessy et al. (2020) discovered that α-solanine and α-chaconine are degraded with α-rhamnosidase, β-glucosidase, and β-galactosidase. Subsequent genomic analysis of *Sodalis* revealed that the strain encoded β-glucosidase and β-galactosidase which could partially degrade α-solanine. Although *Sodalis* lacked α-rhamnosidase, a stepwise removal of glucose and galactose from α-solanine's sidechains was shown to substantially decrease its toxicity (Jensen, Jacobsen, et al., 2009; Jensen, Strobel, et al., 2009). However, it is unclear why the abundance of *Commensalibacter* which was potentially capable of α-solanine and α-chaconine degradation did not increase (**Chapter 5**). On the other hand, black mustard produces isothiocyanates (ITC) to deter insects that are liberated by the glucosinolate-myrosinase defence system (Winde & Wittstock, 2011). Our results demonstrated a substantial increase in *Pantoea* abundance in insects feeding on black mustard, which might suggest its ability to detoxify ITC. A recent study by Shukla and Beran (2020) indicated that *Pantoea* was capable of rapid ITC degradation *in vitro*, and the reintroduction of *Pantoea* to gnotobiotic stem flea beetles *Psylliodes chrysocephala*, restored insects' ability to withstand toxins. Altogether, our results suggested that despite dietary changes, *N. viridula* can maintain core microbes which in return could mediate detoxification of plant toxins. While the stability of the gut microbiome may confer advantages for the insect, such as detoxification, it also could make the insect vulnerable to any disturbances. Eventually, this could destabilize the host microbial community and impair fitness and survival (**Chapter 4**), which could be exploited as an alternative strategy in pest control.

The discovery of the novel *pnm*R opens up opportunities for postharvest decontamination

Approximately 450 plant species within the Fabaceae family and fungi produce toxic nitroglycosides that can be hydrolysed to NPA (Anderson et al., 1993; Baxter et al., 1992). NPA acts as a defence compound that impairs the functioning of the TCA cycle. In recent years, fungal contamination with nitroglycosides. Such as NPA, resulted in food poisoning all over the world leading to serious injuries and substantial economic losses (Birkelund et al., 2021; Liu et al., 1992; Rofiat et al., 2015). One solution that could prevent intoxication is the removal of toxins from

naturally NPA-containing and contaminated products with microbial enzymes. Several bacteria, as described earlier in detail, were reported to be capable of NPA degradation by harbouring either nitronate monooxygenase (NMO) or propionate-3-nitronate monooxygenase (P3NO) in their genomes (Anderson et al., 2000; Nishino et al., 2010). These enzymes have diverse catalytic efficiencies and substrate preferences and were reported to be broadly distributed among living organisms (Anderson et al., 2000; Torres-Guzman et al., 2021). Likewise, *N. viridula* harboured in the gut several NPA-degrading bacteria (**Chapter 6**) and one of them, namely *Pseudomonas* sp. Nvir detoxified NPA with a novel nitronate monooxygenase PNMR (**Chapter 7**).

Already decades ago, studies reported that enzymes of microbial origin could be used to remove toxic constituents; for instance phytate from soybean and wheat or gossypol in cottonseeds (Liener, 1977). Nowadays, enzymatic toxin degradation is a commonly used technique in food processing since it is more specific and environmentally friendly compared to physical and chemical methods (Abraham et al., 2022). Nitronate monooxygenases have broad substrate specificity making them potential preventers of nitroglycoside-related intoxications. These enzymes have already found applications in bioremediation and as pharmaceutical agents and biocatalysts, but further characterization of novel enzymes, such as PNMR, could potentially expand their biotechnological applications to the food processing industry (Torres-Guzman et al., 2021). Xie et al. (2019) recently proposed that insect symbionts might be used as a novel source in antibiotic treatment, enzymatic digestion or plastic biodegradation, which further urges investigating insect-microbe symbiosis.

Future perspectives – exploring insects' microbial communities for pest management

In the work presented in this PhD thesis, we explored insect-microbe symbiosis and bridged knowledge gaps in tri-trophic interactions between *N. viridula*, host plants and associated microbiota. However, several newly emerged research hypotheses remain to be further investigated offering exciting new research opportunities.

The bacteria that can be isolated and grown in the laboratory are just a small fraction of identified microbes (Stewart, 2012) and studying insects' microbial communities often poses challenges due to the limitations of traditional culturing

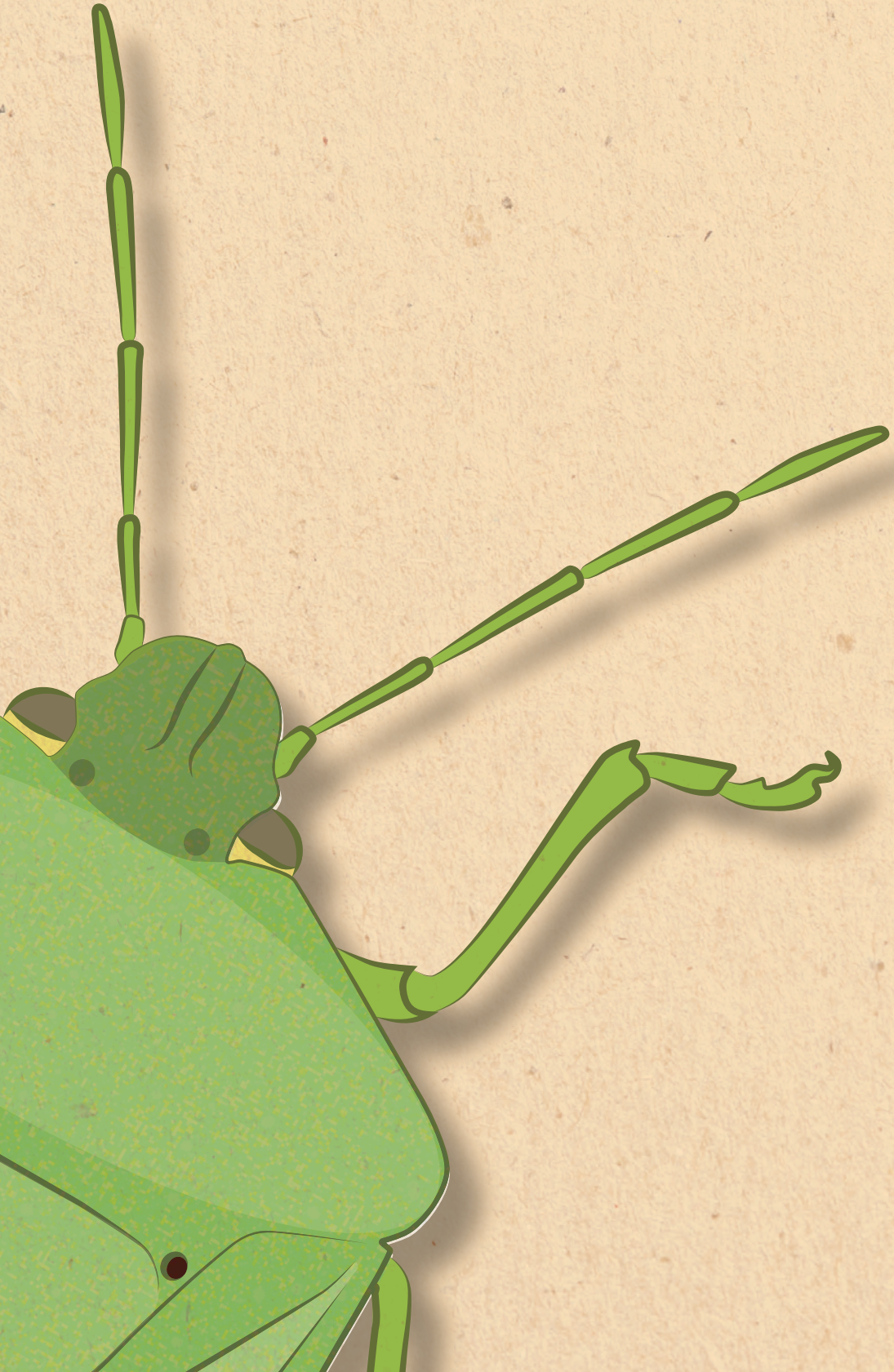
methods. Various methods could advance our knowledge in investigating unculturable insect-associated symbionts, of which some have been applied in this research. Through metagenomics (**Chapter 3**) we gained a comprehensive overview of the gut and salivary glands microbial community, allowing for the exploration of the metabolic roles of two unculturable symbionts, *Pantoea* and *Commensalibacter*. Also, with 16S rRNA gene amplicon sequencing, we determined the gut microbial profile and the effect of dietary changes on its composition (**Chapter 6**). Nevertheless, the active role of *N. viridula*-associated microbes remained unexplored.

Several authors used the metatranscriptomics approach to unveil the active functions of symbionts in insects. Parallel metatranscriptomics analysis of *Altica* flea beetles gut microbiome revealed that the gene expression patterns of insect microbiota were linked to the degradation of secondary plant metabolites, indicating the participation of microbiota in detoxification (Wei et al., 2022). Additionally, Mittapelly (2018) showed that obligate *Ca.* Pantoea carbekii played a crucial role in nutrient provisioning in brown marmorated stink bug *Halyomorpha halys*. A similar investigative approach could be used to study *N. viridula* microbiota, addressing several nurturing questions such as 1) the involvement of core and transient microbes in detoxification (**Chapters 3, 5, 6**) or 2) the role of *Sodalis* sp. Nvir in salivary glands and gut (**Chapter 4**). This would possibly allow us to unravel the functional contribution of *N. viridula* microbiota to the host's ecology and physiology.

Moreover, while *N. viridula* harbours toxin-degrading bacteria in the gut, and the insect itself might be capable of partially or fully detoxifying plant toxins (Li et al., 2007; Winde & Wittstock, 2011). This was recently demonstrated in bean bug *R. pedestris*, which engages in reciprocal detoxification with symbiotic bacteria to detoxify insecticide fenitrothion (Sato et al., 2021). Through metabolomics, the authors investigated the presence of fenitrothion and its degradation products in insect feces. They found that fenitrothion, which is toxic to insects, was degraded by gut symbionts, and that the intermediate metabolite, which is toxic to bacteria, was subsequently excreted by insects. This process enabled the simultaneous maintenance of symbiosis and efficient insecticide degradation. A recent analysis of *N. viridula* M1-M4 gut regions indicated that insects were capable of xenobiotic metabolism (Denecke et al., 2020), thus it would be interesting to use a similar metabolomics approach to determine to what extent *N. viridula* relies on symbiotic associations with microbes to degrade toxic compounds.

Furthermore, recent advances in spatially resolved mass spectrometry (MS) allow researchers to investigate the localization of metabolites involved in host-microbe interactions, including signalling molecules, secondary metabolites or nutrients. It is based on mapping the spatial distribution of analysed molecules within host tissues and symbiont populations allowing to understand the communication between host and microbe (Geier et al., 2020; Taylor et al., 2021). Recently, using spatial MS, it was observed that marine cnidarian hosts could regulate symbiont acquisition and rejection through specific ceramides distributed throughout the gut (Chan et al., 2023). Similarly, exploration of honey bees gut with comparative metabolomics, ^{13}C-labelling and nanoscale secondary ion mass spectrometry determined that the host facilitates the colonization of symbionts by producing specific organic acids (Quinn et al., 2024). Our findings also suggested the presence of a certain sorting mechanism in *N. viridula* which could regulate the structural organization of symbionts in host organs (**Chapter 4**). Moreover, we provided the first insights into collaboration between bacteria (**Chapter 6**). Taken together, it would be intriguing to further explore the concept of microbe-microbe and host-microbe metabolite exchange using novel methods to reveal functional dynamics between host and microbes. This could further broaden our understanding of insect-microbe symbiosis and contribute to the development of alternative pest control strategies.

In past decades, there has been growing interest in exploiting microbes as an alternative pest control strategy, yet only recently have they started to be applied. Among these approaches, the incompatible insect technique has been proven to be an effective approach in pest management. This technique relies on the manipulation of insect microbiota through which male insects are made incapable of reproduction. Another method, paratransgenesis, allows for the genetic modification of the insect gut microbiota by impairing microbes' ability to synthesize essential nutrients. Targeting and deleting detoxifying genes with CRISPR-Cas9 systems could be yet another novel pest control strategy that would eventually contribute to decreasing the spread of resistance phenotype among insects (Pagendam et al., 2020; Ridley et al., 2013; Sander & Joung, 2014; Selle & Barrangou, 2015; Taracena et al., 2015; Zhao et al., 2020). Microbiota-targeting approaches were shown to be effective against aphids, whiteflies or beetles, yet to date, they were not directed towards shield bugs or *N. viridula*. In summary, our research has laid the groundwork regarding *N. viridula* microbiota and its role in overcoming plant defences, which could contribute to the development of tailored pest control methods to eradicate shield bugs in the future.



Appendix

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Data availability

The research in this dissertation has been carried out under the Research Data Management policy of the Radboud Institute for Biological and Environmental Sciences.

Chapter 3:	Data available at https://doi.org/10.5281/zenodo.10630011 Sequencing data available are deposited in the European Nucleotide Archive under Accession Number PRJEB64167.
Chapter 4:	Data available at https://doi.org/10.5281/zenodo.10630011
Chapter 5:	Data available at https://doi.org/10.5281/zenodo.10630011
Chapter 6:	Data available at https://doi.org/10.5281/zenodo.10630011 Sequencing data available are deposited in the European Nucleotide Archive under Accession Number PRJEB70466.
Chapter 7:	Data available at the publisher https://doi.org/10.1128/aem.00719-22 Sequencing data available are deposited in the European Nucleotide Archive under Accession Number PRJEB49126.

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learned a lot. I enjoyed working together with you, and who knows, maybe we will work together again in the future. Fingers crossed for your careers!

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Curriculum vitae

Magda Rogowska-van der Molen was born on September 6th 1994 in Olsztyn, Poland. There she attended XII Academic High School from which she graduated in 2013 and continued to study Biotechnology at the University of Warmia and Mazury in Olsztyn. Magda received her Bachelor of Engineering degree in 2017 and then moved to the Netherlands to pursue a Master's degree in Biotechnology at Wageningen University & Research. During her Master's she found passion in studying microbes in food and agriculture. She conducted her thesis at the Food Microbiology department in Wageningen where she studied ways to develop fermented infant formula. Afterwards, she moved to the Wetsus Research Centre in Leeuwarden, the Netherlands to explore microbes in wastewater treatment plants and the possibilities to use them as natural flocculants. Through her experience in working with microbes, Magda was hired as a PhD Candidate at the Microbiology department at Radboud University in Nijmegen, the Netherlands under Dr. Cornelia Welte with co-supervision of Dr. Robert Jansen. She studied the microbiota of pest insect *Nezara viridula* and her PhD research findings are presented in the current thesis. Soon, she will become a postdoctoral researcher at the University of Cambridge in the United Kingdom, where she will continue research in the field of host-microbe interactions.

Throughout evolution, insects have developed exceptional adaptive capabilities by forming symbiotic relationships with microbes, which are often crucial for their survival. Among shield bugs, such as the Southern green shield bug *Nezara viridula*, mutualistic interactions with bacteria are common and contribute significantly to the insect's ecological success.

N. viridula is a piercing and sucking insect that relies on symbionts for nutrient supplementation. It is a highly invasive species with a broad dietary range across many plant families and its global spread results in significant agricultural losses. Recent studies showed that the shield bug microbiota can manipulate plant defences, affecting pest insect damage potential, however, the role of *N. viridula* microbiota remains unexplored. In this PhD thesis, the knowledge gaps in the insect-microbe symbiosis field were addressed and aimed to understand the role and relationship between *N. viridula* and its associated microbes.

Our research revealed that *N. viridula* has a simple and stable microbiota which participates in digestion, nutrient provision, detoxification and repression of plant toxins which is detrimental to the development of sustainable pest control strategies. This study laid a solid foundation for future research in the area of insect-microbe interactions and their application for agricultural practices and pest management.

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