

Exploring *Drosophila suzukii* microbiome as a new pest biocontrol tool

Rodrigo Filipe Fernandes Narciso

Biologia Celular e Molecular
Departamento de Biologia
2022

Orientador

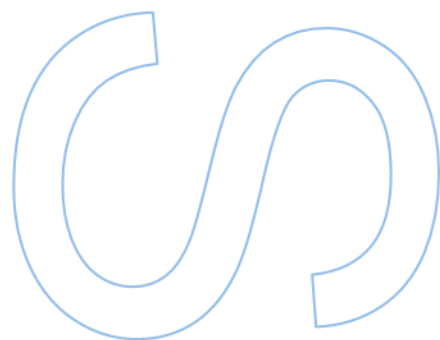
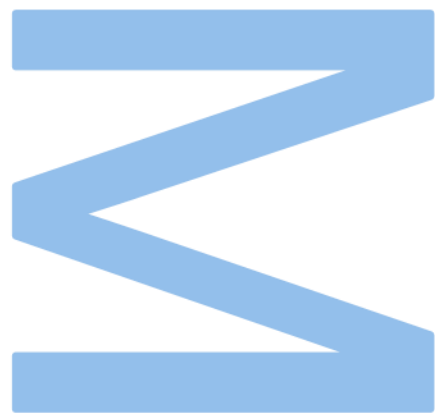
Maria da Conceição Lopes Vieira dos Santos, Professor
Catedrático, Faculdade de Ciências da Universidade do Porto

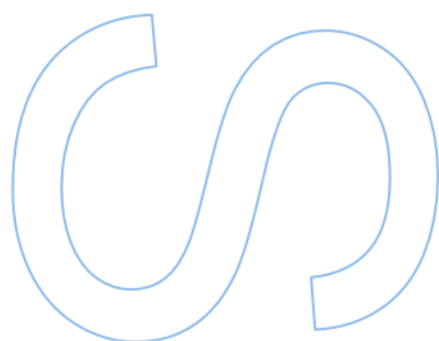
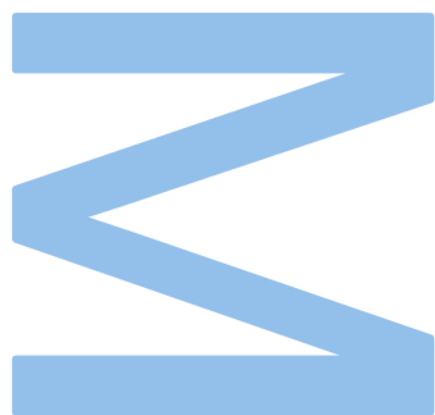
Co-orientador

Luís Manuel Valla Teixeira, PhD, Instituto Gulbenkian de Ciência

Supervisor

Sara Judite Pais Sario, MSc Especialista na área, Faculdade de
Ciências da Universidade do Porto





Exploring *Drosophila suzukii* microbiome as a new pest biocontrol tool

The work presented in this dissertation was carried out at Faculdade de Ciências da Universidade do Porto (FCUP) in the Integrative Biology and Biotechnology (iB₂) Group of Associated Laboratory for Green Chemistry of the Network of Chemistry and Technology (LAQV-REQUIMTE) and Instituto Gulbenkian de Ciência (IGC) in the Host Microorganism Interaction (HMI) Group, and financially supported by Portuguese national funds (FCT/MCTES, Fundação para a Ciência e Tecnologia and Ministério da Ciência, Tecnologia e Ensino Superior) through the projects “DrosuGreen” (reference: PTDC/ASP-PLA/4477/2020) and “A new model system to functionally characterize gut microbiota colonization and interaction with innate immunity” (reference: PTDC/BIA-MIC/31984/2017). This work was also supported by the fellowship PTDC/ASP-PLA/4477/2020.



I dedicate this dissertation

To my beloved family, friends and boyfriend.

Sworn Statement

I, Rodrigo Filipe Fernandes Narciso, enrolled in the Master's Degree in Cellular and Molecular Biology at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

I further declare that all references to other authors fully comply with the rules of attribution and are referenced in the text by citation and identified in the bibliographic references section. This dissertation does not include any content whose reproduction is protected by copyright laws.

I am aware that the practice of plagiarism and self-plagiarism constitute a form of academic offense.

Rodrigo Filipe Fernandes Narciso

30/09/2022

Acknowledgements

For every movie, there is a backstage storyline happening right behind of what meets the eye. In this premiere, I will spare no credits nor gratitude for everyone who followed me and who I followed along the past year. I never thought one could feel such fulfilment, both professionally and personally... What a journey.

First and foremost, I thank Professor Conceição Santos, who let me on this journey at iB₂ and embark on this adventure and trusted me to carry a good work. The expertise and support were fundamental to the place where, not only this dissertation has got to, but myself as a future researcher. Working with Professor Conceição Santos means always stepping up the game and reaching for the higher levels. Truly, an inspiration.

Secondly, I thank my co-supervisor Doutor Luís Teixeira, which welcomed me into HMI and shared his wisdom and expertise with me, bringing new ideas and perspectives, which ultimately improved the final product of this dissertation. Lucky me for working with such an amazing researcher and I will forever carry with me the critical thinking and methodology I learned from Doutor Luís Teixeira.

My panel of supervisors ends with Sara. As much of a *cliché* as it may be, last does not mean least. Thank you for teaching me everything I needed to start my work, for always listening to me and to my most unthinkable questions. I feel so grateful to have learned everything about *Drosophila suzukii* with such an amazing researcher and friend, presenting me with both knowledge and support. You made all this possible at every moment. Thank you for always pushing me upwards and supporting me.

To Rita, an amazing soul whom I followed and taught me so much and integrated me into HMI. It was such a joy to work alongside an amazing person and a positive energy, while learning loads and loads of new stuff. Thank you so much Rita!

Another big shoutout goes to Rafael, an incomparable soul, energy, mind, colleague and friend. No 'thank you' is enough to compensate for all the help and care you provided me with. Not only me, everyone! Your sense of duty to support and help was a big foundation of this work. You would never let this fail. I wish one day, give back to you what you gave me and if I'm not able to, I hope the world does it for me.

Work is not only stressing and obsessing. It is also about sharing moments of joy and Laura was always up to make me laugh at the most and least stressful moments. You made my journey a little (ok, a lot) brighter than it could have been possibly. Lucky to have had such an amazing soul and warm heart around me.

Anicia, a matching energy. You set fire to the room (in the best way possible). Around you, everything becomes lighter. I feel so grateful to have come across with such an amazing person, with such an amazing heart. It's so calming to have someone beside us who can make us feel understood and share the same feelings about most. You're that person, thank you so much!

Finally, I could not neglect the rest of my colleagues at iB₂ and HMI. You were all part of this journey and played your part in shaping my work. A special thank you to Juliana, Paulo, Leandro, Nuno, Marino, João e Emil at iB₂ and to Catarina, Pedro, Teresa, Beatriz, Rafael, Nelson, Migla, André and Miguel at HMI. I would also like to thank my colleagues at MDE, led by Professor Fernando Tavares, especially Leonor and Kayla, for the kind help.

Moving to my friends... My chosen family.

When I moved to Porto, I could never imagine the amount of good, kind, caring and fun people I would meet. You were the one who all lifted my spirit up. Who illuminated my mind when my perception was nothing but dark. To David, the kindest and funniest soul to walk on Earth. To Rafael, who never failed to support me, my roomie, my very first companion here. To Sara, the friend we all wish to have, you can always put a smile on me. To Edu, a wild energy with such a warm heart, you turn everything brighter. To Francisco, your presence is a joy I never want to miss. To Isa, such a unique energy I envy. My Singelers, love y'all.

Everyday, I believe that life will by itself lead me to people I did not know I needed and so it made me meet Maria and Jota. Maria, the greatest storyteller I know, it's impossible to remain indifferent to your presence and your speech, you carry the right words all the time. Jota, you are made of kindness and selflessness. You bring a kind of peace I never knew before. Thank you both for welcome me into your lives.

Patrícia, my friend for the longest time. We met so young and it's so fulfilling to watch us grow together. I am so proud of our friendship and how far we've come. Thank you for the time you always have for me.

To Nuno, despite the long distance, I will always spare a place for you in my heart. Friendship looks like you.

À família. Às pessoas que eu há mais tempo conheço e que me acompanharam desde o segundo zero. O agora não existia sem vocês. O vosso orgulho em mim é um

combustível que alimenta a minha vontade de continuar e almejar para mais. Isto é por vocês.

Mãe, o meu pilar, a minha vida. Os teus ensinamentos, a educação que me deste, os valores, a dedicação e o amor tornaram-me na pessoa que sou hoje. Se aqui cheguei, foi graças a ti. Amo-te para sempre.

Pai, o meu exemplo. Inspiração e vontade de querer sempre mais. És o motor que me permite manter a cabeça erguida e avançar para o sucesso. Isto também é teu. Amo-te muito, pai.

Mano, o espelho. És o espelho daquilo que um dia desejo ser. Apoio-me nas tuas conquistas. E as minhas, são as tuas também. Amo-te, maninho.

Um agradecimento especial à minha Tia Joana, um exemplo de força e dedicação. Nunca te esqueceste de mim. Às minhas Avós, pelo apoio incondicional. Ao meu Avô, a quem tive de dizer Adeus no último ano. Tantas mais pessoas para agradecer na minha família. Um obrigado a todos.

Zé, my everyday foundation. This journey was a rollercoaster that I had the luck to share beside you. When I stressed, you hugged me... And everything was calm. When I cried, you cheered me with your voice... And everything was fine. When I was happy, we shared smiles... And everything was even better. This was not an easy ride, but with you by my side at every single moment, it was one that I would not trade. Thank you. I love you.

Resumo

A praga *Drosophila suzukii* é uma ameaça atual para produtores de frutos vermelhos em diversos continentes. Esta espécie invadiu os continentes Europeu e Americano em 2008, e rapidamente se espalhou por diversos países, levando a perdas devastadoras de culturas e consequências económicas. Em 2012, a praga *D. suzukii* foi reportada, pela primeira vez, em Portugal, nas regiões de Odemira e Algarve, invadindo, inevitavelmente, as regiões Centro e Norte do país.

As estratégias de mitigação desta praga ainda se baseiam na aplicação de produtos químicos, como a espinosina. Contudo, existe uma crescente preocupação e criticismo quanto ao uso de pesticidas devido aos perigos ambientais e riscos de saúde pública, dado o seu espectro de ação largo, aquisição de resistência e presença residual em produtos comercializados.

Para responder aos danos causados por pragas de forma mais sustentável, o controlo biológico é de grande interesse pelas comunidades científica e agroindustrial, dado que pode ser aplicado em programas de gestão integrada de pragas (IPM), de modo a criar estratégias de controlo eficientes e ambientalmente sustentáveis. Particularmente, uma destas estratégias assenta no uso de bactérias entomopatogénicas para reduzir os danos e incidência de pragas.

Seguindo estas linhas, este trabalho tem o intuito de explorar novas estratégias de controlo da praga *D. suzukii*, tirando vantagem do potencial de microrganismos e do microbioma. Como tal, o primeiro objetivo é caracterizar a microbiota intestinal de populações portuguesas de *D. suzukii*, de modo a decifrar o microbioma *core* e bactérias naturais associadas a esta espécie. O segundo objetivo é analisar a atividade entomopatogénica de bactérias e os mecanismos moleculares de patogenicidade ou resistência. Por fim, o terceiro objetivo é revelar relações simbióticas com bactérias com base na sua capacidade de colonizar o trato intestinal de *D. suzukii* através do desenvolvimento de uma nova metodologia.

A primeira tarefa foi conseguida através de uma análise metagenómica, utilizando uma plataforma de sequenciação de nova geração (NGS) e por isolamento de bactérias. Ambos os métodos foram aplicados em moscas capturadas em campo e eclodidas em framboesa e mirtilo, de diferentes pomares portugueses, e em moscas de laboratório. O filo Proteobacteria foi identificado como o mais prevalente em associação com *D. suzukii* e a família Acetobacteraceae dominou o microbioma *core*. Amostras de

machos *D. suzukii* desenvolvidos em framboesa, fêmeas desenvolvidas em mirtilo e moscas de laboratório demonstraram distanciamento em diversidade bacteriana em relação às restantes amostras. O género *Frigoribacterium* é uma novidade, uma vez que foi isolado pela primeira vez em associação com *D. suzukii* em quase todos os pomares, tanto em machos como fêmeas.

De seguida, duas bactérias putativamente entomopatogénicas isoladas a partir de amostras de *D. suzukii*, *Microbacterium thalassium* e *Providencia* sp., foram testadas quanto à sua atividade inseticida, mostrando nenhuma mortalidade a curto prazo. Apesar de nenhum efeito inseticida ter sido demonstrado, mecanismos moleculares de imunidade foram avaliados em diferentes tempos de exposição a ambas as bactérias, para traçar um perfil de mecanismos de resistência imunitária a estas bactérias. As vias de imunidade Imd e Toll foram estudadas através da quantificação da expressão de transcritos. A expressão de *duox*, um regulador da microbiota intestinal, mostrou ser regulado positivamente como resposta à infeção e a via Toll mostrou ser regulada positivamente apenas após 24 horas de exposição. Além disso, numa análise de componentes principais (PCA), foi revelada uma resposta dependente do sexo, uma vez que as fêmeas mostraram uma resposta mais antecipada através de péptidos antimicrobianos contra *Providencia* sp., associada com a via Imd. Contrariamente, machos revelaram ser mais suscetíveis a *M. thalassium*.

Por fim, um método de estudo da capacidade de proliferação de bactérias foi desenvolvido através da otimização de um protocolo anterior para *Drosophila melanogaster*. Este método permite a avaliação da capacidade de colonização de bactérias, isoladas de *D. suzukii*, após ingestão oral por moscas axénicas. Após estabelecer a metodologia, *Leuconostoc pseudomesenteroides* mostrou proliferação no intestino de fêmeas de *D. suzukii* mas não no de machos. Este resultado, não só fornece um controlo positivo para um ensaio, que até agora faltava, mas também demonstra novamente uma dinâmica dependente do sexo. Para além disso, *Acetobacter thailandicus*, isolada de amostras de *D. suzukii* de diferentes regiões, foi testada usando o mesmo ensaio e os dados demonstram um comportamento diferente entre isolados, sugerindo uma capacidade de colonização dependente da estirpe.

Estes dados respondem aos objetivos delineados e apoiam a ideia do microbioma como uma estrutura com potencial para ser manipulado para o controlo da praga *D. suzukii*. A caracterização da comunidade bacteriana de populações portuguesas de *D. suzukii* indicou uma forte associação com a bactérias da família Acetobacteraceae e revelou um novo género associado com esta espécie, indicando possivelmente um

microbioma *core* específico de populações portuguesas. Ademais, esta espécie demonstra uma dinâmica dependente de sexo e de estirpe quanto a respostas moleculares de imunidade e estabelecimento de relações simbióticas, que devem ser tidas em conta para o desenvolvimento de estratégias eficientes para controlo de praga.

Palavras-chave: Bactérias entomopatogénicas, Bactérias simbióticas, Controlo biológico, Controlo sustentável de pragas, *Drosophila suzukii*, Ensaio de proliferação, Expressão de genes, Metagenómica, Microbioma

Abstract

The *Drosophila suzukii* pest is a current threat to red fruits and berries producers across multiple continents. This species has invaded the European and American continents in 2008, swiftly spreading across different countries, leading to devastating crop loss and economic consequences. In 2012, *D. suzukii* was reported for the first time in Portugal in the regions of Odemira and Algarve, inevitably invading the Centre and North regions of the country.

Pest mitigation strategies still rely on the application of chemical products, such as spinosyns. However, there is a growing concern and criticism of the use of pesticides due to environmental hazards and public health risks, for their broad-spectrum range, resistance acquisition, and residual presence in commercialized products.

To respond in a more sustainable way to pest damage, biological control is of great interest to the scientific and agroindustry communities as it may be applied in Integrated Pest Management (IPM) programs to create efficient and environmentally friendly pest control strategies. One particular strategy relies on the use of entomopathogenic bacteria towards the pest to reduce the incidence and damage.

Following these lines, the work present in this dissertation aims to explore new strategies for *D. suzukii* pest control by harnessing the potential of microorganisms and the microbiome. To achieve that, the first objective was to characterize the gut microbiota of Portuguese populations of *D. suzukii* to decipher the core microbiome and natural bacteria associated with this species. The second one was to screen the entomopathogenic activity of bacteria and the molecular mechanisms underlying the pathogenicity or resistance. Finally, the third objective was to disclose symbiotic relationships with bacteria based on their colonizing ability in the gut of *D. suzukii* by developing a new methodology.

The first task was accomplished by resorting to metagenomic analysis, using a next-generation sequencing (NGS) platform, and by the isolation of bacteria. Both methods were applied to field-collected, raspberry, and blueberry-emerged flies from different Portuguese orchards and laboratory-reared flies. Proteobacteria was identified as the most prevalent phyla associated with *D. suzukii* and the Acetobacteraceae family dominated the core microbiome. Male samples of *D. suzukii* developed in raspberries, females developed in blueberries and laboratory-reared flies demonstrated a distance in bacterial diversity from the remaining samples. *Frigoribacterium* is a novel genus

associated with *Drosophila* and was isolated from almost all orchards in both male and female flies.

Secondly, two putative entomopathogenic bacteria isolated from *D. suzukii* samples, *Microbacterium thalassium*, and *Providencia* sp. were tested for their insecticidal activity by orally infecting *D. suzukii* flies, displaying no short-term mortality. Although no insecticidal effect was disclosed, molecular mechanisms of immunity were assessed at different times of exposure to both bacteria, to profile resistance immunity mechanisms to these bacteria. The Imd and Toll immunity pathways were studied by quantification of transcript expression. The expression of *duox*, a gut microbiota regulator, was found to be upregulated in response to infection and the Toll pathway was found to be upregulated only at 24 hours of exposure. Besides, with a further principal component analysis (PCA), a sex-dependent response to infection was disclosed, as females displayed an earlier antimicrobial peptide response to *Providencia* sp., associated with the Imd pathway. Contrarily, males revealed more susceptibility to *M. thalassium*.

Finally, a method to study the proliferation capacity of bacteria was developed by optimizing a previous protocol for *Drosophila melanogaster*. This method enables the assessment of the colonizing ability of bacteria, naturally isolated from *D. suzukii*, after oral ingestion by axenic *D. suzukii*. After establishing the methodology, *Leuconostoc pseudomesenteroides* was found to proliferate in the gut of *D. suzukii* female flies, but not on male flies. This result not only provides a positive control for this assay which, until now, was lacking but again, a sex-dependent dynamic. Moreover, *Acetobacter thailandicus* isolated from *D. suzukii* samples from different regions was assessed using the same assay and the data displayed different behaviour between isolates, suggesting a strain-dependent colonizing ability.

These data respond to the delineated objectives and support the microbiome as a potential manipulable structure to control the *D. suzukii* pest. The characterization of the bacterial community of Portuguese populations of *D. suzukii* indicates the strong association with bacteria from the Acetobacteraceae family and revealed a new genus associated with this species, possibly indicating a core microbiota specific to Portuguese populations. Moreover, this species displays sex-dependent and strain-dependent dynamics in immune molecular response and establishment of symbiotic relationships, which should be taken into account for the development of efficient pest control strategies.

Keywords: Biological control, *Drosophila suzukii*, Entomopathogenic bacteria, Gene expression, Metagenomics, Microbiome, Proliferation Assay, Sustainable pest control, Symbiotic bacteria

Table of Contents

List of Tables	viii
List of Figures	ix
List of Abbreviations	xiii
I. Chapter 1: Gut microbiome characterization and manipulation to control <i>Drosophila suzukii</i> pest.....	1
1. Introduction	1
2. <i>Drosophila suzukii</i> gut bacterial microbiome characterization.....	5
2.1. Acetic Acid Bacteria	5
2.2. Enterobacteriaceae	6
2.3. Lactic Acid Bacteria.....	7
2.4. Implications	8
3. Integrated Pest Management (IPM) Strategies for SWD Pest Control	16
3.1. A synopsis of current control practices	16
3.2. Manipulating microbiomes as an emerging control strategy	18
3.2.1. Entomopathogens	18
3.2.2. <i>Drosophila</i> immunity	20
3.2.3. Disrupting the microbiome homeostasis	23
4. Final Remarks	23
References.....	25
I.II. Hypothesis & Objectives	42
II. Chapter 2: Unveiling Portuguese <i>Drosophila suzukii</i> microbiome assembly: do sex and food supply matter?	43
Abstract.....	43
1. Introduction	43
2. Materials and Methods	46
2.1. <i>Drosophila suzukii</i> Collections.....	46
2.2. Bacterial Isolation and Identification	46

2.3. Identification of Bacterial Isolates	47
2.4. Genomic DNA Collection for Metagenomics	47
2.5. NGS 16S rRNA Metagenomics	47
2.6. Sequencing Data Analysis	47
2.6. Microbial Community Structure and Alpha and Beta Diversity Analysis.....	48
3. Results	48
3.1. Sequencing Data and Quality	48
3.2. Bacterial Community of Wild Female <i>Drosophila suzukii</i>	49
3.4. Bacterial Communities of fruit-emerged <i>Drosophila suzukii</i> individuals	50
3.5. Bacterial Community of Laboratory-reared Male <i>Drosophila suzukii</i>	52
3.2. Species Richness and Alpha Diversity of Bacterial Communities.....	53
3.6. Beta-Diversity and Distance Analysis of Microbial Communities	55
4. Discussion.....	57
References.....	62
Supplementary Materials.....	70
III. Chapter 3: <i>Drosophila suzukii</i> displays a sex-dependent immune response to <i>Microbacterium thalassium</i> and <i>Providencia</i> sp. infection	71
Abstract.....	71
1. Introduction	71
2. Materials and Methods	74
2.1. <i>D. suzukii</i> Collection and Rearing.....	74
2.2. Sample Processing for Bacterial Isolation	74
2.3. Identification of Bacteria	74
2.4. Oral Infection.....	75
2.5. Collection of Orally Infected Flies and Colony Forming Units (CFU) Count	75
2.6. Survival Assays.....	76
2.7. RNA Extraction and cDNA synthesis.....	76
2.8. Gene Expression by Real-Time Quantitative PCR (RT-qPCR).....	76
2.9. Statistical Analysis	77

3. Results.....	78
3.1. Bacterial Levels Are Similar at 6 and 24 Hours of Exposure.....	78
3.2. Non-Lethal Bacteria Affect <i>duox</i> Expression	78
3.3. <i>atf2</i> is Differentially Regulated According to Exposure Time.....	80
3.4. Females Disclose an Immune Response to <i>Providencia</i> sp. and Males to <i>Microbacterium</i> sp.	81
3.5. Multivariate Analysis Discloses Immune Response Associated with Bacterial Infection	82
4. Discussion.....	83
References.....	87
IV. Chapter 4: Assessing the proliferation capacity of putative stable gut bacteria of <i>Drosophila suzukii</i>	94
Abstract.....	94
1. Introduction	94
2. Materials and Methods	96
2.1. <i>D. suzukii</i> Collection and Rearing.....	96
2.2. Sample Processing for Bacterial Isolation	97
2.3. Identification of Putative Stable Bacteria	97
2.4. Establishment of a Protocol to Generate Axenic <i>D. suzukii</i>	98
2.5. Establishment of gnotobiotic associations and Proliferation Assay	98
2.5.1. Feeding Conditions.....	98
2.5.2. Proliferation Time	99
2.6. Statistical Analysis	99
3. Results.....	100
3.1. Optimal Feeding Conditions	100
3.2. Influence of the Reingestion Cycle on the Proliferation.....	100
3.3. Proliferation of <i>L. pseudomesenteroides</i> and <i>A. thailandicus</i> in <i>D. suzukii</i> : Influence of Sex and Bacterial Strain	103
4. Discussion.....	104
References.....	109

V. Chapter 5: General Discussion	115
1. Characterization of the Microbiome of Portuguese <i>Drosophila suzukii</i> Populations	115
2. Sex-Dependent Response of <i>Drosophila suzukii</i> to Bacterial Infections	117
3. Methods to Study Symbiotic Bacteria in <i>Drosophila suzukii</i>	118
4. Final Remarks	119
V.II. Future Perspectives.....	121
References.....	122

List of Tables

Table I-1 - Bacteria associated with <i>Drosophila suzukii</i> . Compilation of species and genera of all bacteria identified in the gut of <i>D. suzukii</i> according to different publications, alongside with the method of identification, the developmental stage in which the bacteria were identified, the effect of bacteria when <i>D. suzukii</i> is infected with them, the source of <i>D. suzukii</i> and the morphotype.....	10
Table II-1 - Sequencing data and quality control stats. Number of raw reads, raw tags, clean tags and effective tags (after cleaning and filtering) by sample alongside with average and sum for all samples. Average length of the effective tags, percentage of tags with quality score equal to or above 20 (Q20) and percentage of effective tags according to the number of raw reads. Number of annotated tags (assigned to a taxon), unannotated tags (with no assignment), unique tags (number of tags with a frequency of 1 unique to that sample) and number of OTUs per sample. Sample names: CP21F (Castelo de Paiva, 2021, Females), G21F (Vila Nova de Gaia, 2021, Females), G21M (Vila Nova de Gaia, 2021, Males), OAZ21F (Oliveira de Azeméis, 2021, Females), OAZ21M (Oliveira de Azeméis, 2021, Males), LabM (Laboratory-reared, Males).....	49
Table II-2 – Isolates from wild-caught <i>Drosophila suzukii</i> . Different species, genera or families identified from wild-caught <i>D. suzukii</i> from Castelo de Paiva, 2021 (CP21F) according to sex (male and female) and in larva.	50
Table II-3 - Isolates from fruit-emerged adults of <i>Drosophila suzukii</i> . Different species, genera or families identified from fruit-born <i>D. suzukii</i> collected from Oliveira de Azeméis, 2021 (OAZ21), Santa Maria da Feira, 2021 (SMF21) and Vila Nova de Gaia, 2021 (G21), Portugal 2021 according to sex (male and female).	52
Table II-4 - Isolates from laboratory-reared <i>Drosophila suzukii</i> . Different species or genera were identified from laboratory-reared <i>D. suzukii</i> according to sex (male and female).....	53
Table II-5 - Community diversity and richness indices. Number of observed species, alongside with Shannon and Simpson indices, representing diversity, and Chao1 and ACE indices representing richness of the different samples.....	55
Table SII-6 - Samples of <i>D. suzukii</i> used for identification of bacterial communities by isolation and metagenomics.....	70
Table III-1 - Primers used for transcripts expression analysis by RT-qPCR	77

List of Figures

Figure I.1 - Structure of the gut of <i>Drosophila</i> and interaction with its microbial community. The gut of <i>Drosophila</i> is structured in three main sections, the foregut, followed by the midgut and ending at the hindgut, comprising other secondary structures, such as the crop, cardia and copper cell region. Across the gut, <i>Drosophila</i> establishes interactions with microbes, which may vary according to its nature. Some microbes interact by processing molecules and influence the availability of nutrients for the host. Microbes also play a role in activating pathways, such as the JNK pathway, which, in turn, activates the JAK/STAT pathway in intestinal stem cells (ISCs), promoting proliferation and gut repair. Between the microbial community, there is an interchange of metabolites, forming a network of interaction and metabolic pathways. Finally, the microbes trigger immunity pathways, such as the immune deficiency (Imd) pathway, which regulates the microbiota in the gut by the release of antimicrobial peptides (AMPs) and reactive oxygen species (ROS).....	2
Figure I.2 - <i>Drosophila suzukii</i> pest. (A) <i>D. suzukii</i> female adults are able to lay eggs inside ripening or ripen red fruits and berries. (B) <i>D. suzukii</i> male adults are distinguished for their dark spot in the first vein of both wings.....	4
Figure I.3 - Global distribution of <i>Drosophila suzukii</i> . Distribution of <i>D. suzukii</i> across the world according to the data published by the European and Mediterranean Plant Protection Organization at 26 th September 2022: https://gd.eppo.int/taxon/DROSSU/distribution	4
Figure I.4 - Biological control methods of the <i>Drosophila suzukii</i> pest. Strategies of biological control comprising different agents, such as predators, parasitoids, entomopathogenic nematodes and microorganisms (fungi and bacteria). These agents may be applied according to different methods and act upon different developmental stages. Figure adapted from Lee <i>et al</i> (2019) ¹⁵¹	18
Figure I.5 - The Imd and Toll pathways in <i>Drosophila</i> . The Toll pathway is activated mainly by Gram-positive bacteria and fungi. The peptidoglycan receptor proteins, PGRP-SA, GNB1 and GNB3 are responsible for the activation of the modular serine protease (modSP) that activate the Spätzle-processing enzyme (SPE), which, in turn, cleaves Spätzle. Spätzle is a ligand of the Toll receptor, eliciting a signalling response in the cell, via the activation of Myd88, Tube, Pelle and Dif, a transcription factor, responsible for the expression of Drosomycin. The Imd pathway is mainly activated by Gram-negative bacteria, recognized by PGRP-LC and PGRP-LE, starting a signalling cascade by activating Imd, which, in turn, activates Tak1 and IKK, enabling the	

translocation of phosphorylated Relish, a transcription factor, to the nucleus and activating the expression of Dipterecin. Another ramification of the Imd pathway leads to the activation of Mekk1. However, the activation of this MAPKKK can be accomplished independently of Imd activation, by the recognition of pathogen-derived metabolites. Mekk1 will then lead to the activation of the MAPK p38, culminating in the transcription of the Dual-oxidase by the Atf2. Figure retrieved from Buchon <i>et al</i> (2018) ¹⁶⁷	21
Figure II.1 - Relative abundance of genera in wild female <i>Drosophila suzukii</i> . Top 10 most representative genera in Castelo de Paiva, 2021 female (CP21F) sample.	50
Figure II.2 - Relative abundance of genera in fruit-born <i>Drosophila suzukii</i> . (A) Top 10 most representative genera in Vila Nova de Gaia, 2021 (G21) and Oliveira de Azeméis (OAZ21) according to sex (male, M and female, F). (B) Venn diagram representing the number of shared and unique OTUs between raspberry-born (OAZ21) and blueberry-born (G21) group samples.	51
Figure II.3 - Relative abundance of genera in laboratory-reared <i>Drosophila suzukii</i> . Top 10 most representative genera in laboratory-reared male <i>D. suzukii</i> (LabM) samples.	53
Figure II.4 - Rarefaction curves for observed species number. Number of OTUs retrieved according to the number of sequences from the different samples.	54
Figure II.5 – Beta-diversity analysis of the microbial communities. Principal Coordinate Analysis (PCoA) regarding the Unweighted (A) and Weighted (B) UniFrac calculations for all samples, displaying the distance between all samples. (C) Pairwise dissimilarity coefficients obtained by Unweighted (top values) and Weighted (bottom values between parentheses) UniFrac measurements.	56
Figure II.6 - Flower diagram of shared and unique OTUs. Represents the unique OTUs of each sample and the core bacterial OTUs common to all samples.	57
Figure III.1 - Bacterial levels (log CFU/fly) after 6 and 24 hours of exposure. Mean log CFU/fly after 6 and 24 hours of exposure (color coded) to <i>M. thalassium</i> and <i>Providencia</i> sp. in female (A) and male (B) flies. Different lowercase letters represent significant differences ($p < 0.05$) between conditions in 6 hours of exposure and different uppercase letters represent significant differences ($p < 0.05$) between conditions in 24 hours of exposure.	78
Figure III.2 - Expression of Imd and Toll pathways target genes. Relative normalized mRNA expression of <i>duox</i> (A, B), <i>dptA</i> (C, D) and <i>drs</i> (E, F) in female (A, C, E) and male flies (B, D, F). Error bars represent SEM of means. Different lowercase letters represent significant differences ($p < 0.05$) between conditions in 6 hours of exposure and different uppercase letters represent significant differences ($p < 0.05$) between	

conditions in 24 hours of exposure and * represents significant differences ($p < 0.05$) between 6 and 24 hours on the same condition.	79
Figure III.3 - Expression of Imd and Toll pathways transcription factors. Relative normalized mRNA expression of <i>atf2</i> (A, B) and <i>dif</i> (C, D) in female (A, C) and male flies (B, D). Error bars represent SEM of means. Different lowercase letters represent significant differences ($p < 0.05$) between conditions in 6 hours of exposure and different uppercase letters represent significant differences ($p < 0.05$) between conditions in 24 hours of exposure and * represents significant differences ($p < 0.05$) between 6 and 24 hours on the same condition.	80
Figure III.4 - Expression of Imd and Toll pathways components. Relative normalized mRNA expression of <i>mekk1</i> (A, B), <i>imd</i> (C, D) and <i>spz</i> (E, F) in female (A, C, E) and male flies (B, D, F). Error bars represent SEM of means. Lowercase letters represent significant differences ($p < 0.05$) between conditions in 6 hours of exposure and uppercase letters represent significant differences ($p < 0.05$) between conditions in 24 hours of exposure and * represents significant differences ($p < 0.05$) between 6 and 24 hours on the same condition.	81
Figure III.5 - Principal Component Analysis of gene expression of Imd and Toll pathways components by oral infection for 6 and 24 hours. Biplots of females' (A) and males' (B) gene expression associate loadings of <i>duox</i> , <i>dptA</i> , <i>drs</i> , <i>atf2</i> , <i>dif</i> , <i>mekk1</i> , <i>imd</i> and <i>spz</i> and PC Scores of <i>M. thalassium</i> and <i>Providencia</i> sp. at 6 and 24 hours of exposure.....	82
Figure IV.1 – Axenic flies and proliferation assay experimental design.	99
Figure IV.2 - Quantification of CFU/fly according to different feeding conditions. Quantification of CFU/fly according to different feeding conditions. Tested 6 hours feeding (6H), 4 hours feeding (4H) and overnight starvation + 4 hours feeding (ONS + 4H) in females (A) and males (B). Dots and red bar represent a single fly and the mean, respectively. Line shows the minimal detection limit of log CFUs = $\log_{10} 5$	100
Figure IV.3 - Quantification of CFU/fly after 20 hours of proliferation with chasers. Bacterial levels after 20 hours of proliferation and comparison with chaser flies in female and male flies exposed to <i>A. thailandicus</i> (A and C) and <i>L. pseudomesenteroides</i> (B and D). Dots and red bar represent a single fly and the mean, respectively. Line shows the minimal detection limit of log CFUs = $\log_{10} 5$	101
Figure IV.4 - Quantification of CFU/fly after 16 hours of proliferation with chasers (<i>L. pseudomesenteroides</i>). Bacterial levels after 16 hours of proliferation and comparison with chaser flies in female (A) and male (B) flies exposed to <i>L. pseudomesenteroides</i> . Different shapes represent different replicates. Dots and red bar represent a single fly	

and the mean, respectively. Line shows the minimal detection limit of log CFUs = log ₁₀ 5.	102
Figure IV.5 - Quantification of CFU/fly after 16 hours of proliferation (<i>A. thailandicus</i>). Bacterial levels after 16 hours of proliferation and comparison with chaser flies in female (A) and male (B) flies exposed to <i>A. thailandicus</i> . Dots and red bar represent a single fly and the mean, respectively. Line shows the minimal detection limit of log CFUs=log ₁₀ 5.	103
Figure IV.6 - Quantification of CFU/fly after 16 hours of proliferation (<i>A. thailandicus</i> (CP21)). Bacterial levels after 16 hours of proliferation and comparison with chaser flies in female flies exposed to <i>A. thailandicus</i> . Dots and red bar represent a single fly and the mean, respectively. Line shows the minimal detection limit of log CFUs = log ₁₀ 5.	104
Figure IV.7 - Possible model for the establishment of a strain and sex-specific relationship in <i>D. suzukii</i> populations. A colonizing species of bacteria present in the environment (blue rods) is ingested by a population of <i>D. suzukii</i> (male and female with different colors) and establishes a symbiotic relationship, which can evolve into a specific strain and sex-dependent link (color match between bacteria and male/female flies).....	107

List of Abbreviations

AMP	ANTIMICROBIAL PEPTIDE
bp	BASE PAIR
DNA	DEOXYRIBONUCLEIC ACID
EPPO	EUROPEAN AND MEDITERRANEAN PLANT PROTECTION ORGANIZATION
EtOH	ETHANOL
FCUP	FACULTY OF SCIENCES UNIVERSITY OF PORTO
iB2	INTEGRATIVE BIOLOGY AND BIOTECHNOLOGY
IGC	INSTITUTO GULBENKIAN DE CIÊNCIA
IPM	INTEGRATED PEST MANAGEMENT
LB	LYSOGENY BROTH
MRS	DE MAN, ROGOSA AND SHARPE
NCBI	NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION
NGS	NEXT GENERATION SEQUENCING
OD ₆₀₀	OPTICAL DENSITY AT 600nm
PBS	PHOSPHATE BUFFERED SALINE
PCA	PRINCIPAL COMPONENT ANALYSIS
PCR	POLYMERASE CHAIN REACTION
PGRP	PEPTIDOGLYCAN RECOGNITION PROTEIN
RNA	RIBONUCLEIC ACID
ROS	REACTIVE OXYGEN SPECIES
rRNA	RIBOSSOMAL RNA
RT-qPCR	REAL-TIME QUANTITATIVE PCR
SWD	SPOTTED-WING DROSOPHILA
TF	TRANSCRIPTION FACTOR

I. Chapter 1: Gut microbiome characterization and manipulation to control *Drosophila suzukii* pest

1. Introduction

Across all metazoan taxa, microbial communities establish social relations with hosts composing the holobiont or metaorganism¹. The host-microbe interactions condition the behaviour, adaptation, and survival of the host, and when the interactions involve symbiotic relations, also optimal environmental conditions are provided by the host to the symbiont microbial population (as commensal and mutualistic symbiosis)^{2–6}.

The host's microbiome comprises the core (stable and in general commonly present in all individuals of the species) and transient microbial populations^{7,8}. Particularly the transient microbiota is dependent on several shaping factors, such as age, developmental stage of the host, its physiology, genetic background, diet, and environment^{9–12}.

Invertebrate animals, such as insects, also establish tight relationships with their microbiota, particularly that of the gut, as it promotes the host's fitness, in response to the increased availability of some nutrients, and mitigate environmental stress^{13–15}. Understanding these interactions is relevant when studying insect pests, as taking advantage of these tight links opens new horizons to more sustainable and integrated management strategies: for instance, reducing egg viability by exchanging symbiotic bacteria¹⁶, altering the host's stress tolerance using specific bacterial strains¹⁷ or antagonism towards the microbial consortium¹⁸.

The gut microbiome of “vinegar flies”, genus *Drosophila* (Diptera: Drosophilidae), particularly of *Drosophila melanogaster* as a model organism, has been widely studied, as it enables a high-throughput experimental design for microbiome research with low ethical concerns when compared to other *in vivo* models¹⁹. Although relatively simple in composition, the microbiota of *D. melanogaster* acts and interacts in the gut – which is structured by the foregut, midgut, and hindgut. This interaction promotes maintenance of homeostasis^{20,21} by eliciting metabolic signalling pathways²², shielding the host from dangerous compounds (such as pesticides)²³, regulating nutritional indexes²⁴, increasing stress tolerance²⁵, establishing a beneficial and regulated network of metabolites exchange^{20,26}, and mediating neuronal activity and behavior^{27,28} (Figure

I.1). However, commensal and innocuous participants of this microbial community can be just as quick to disrupt the gut homeostasis and become pathogenic to the fly^{26,29}. These new concepts and tools for studying the microbiome have already been developed and established for this particular model. Now, the scientific community may use these tools to further use the microbiome as an applied tool for the development of new technologies, namely to study insect pests.

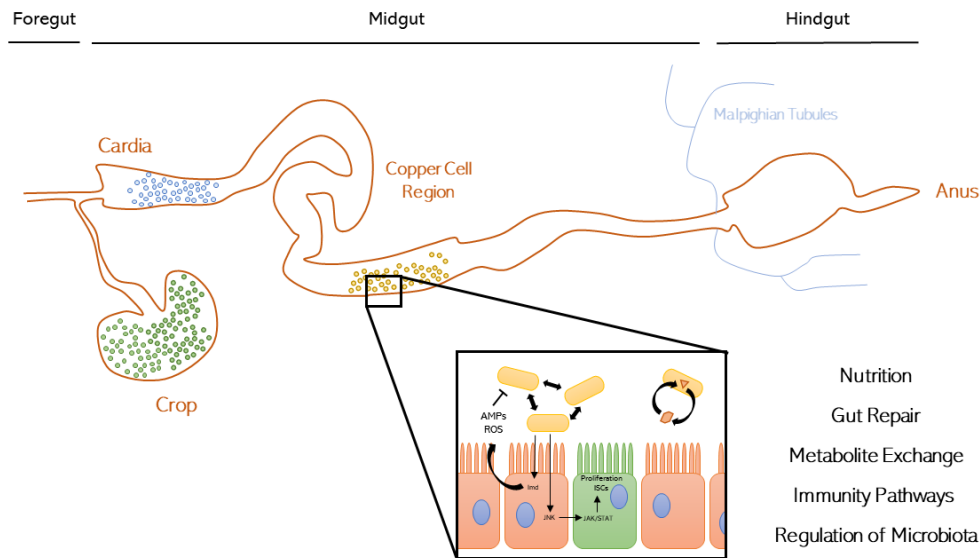


Figure I.1 - Structure of the gut of *Drosophila* and interaction with its microbial community. The gut of *Drosophila* is structured in three main sections, the foregut, followed by the midgut and ending at the hindgut, comprising other secondary structures, such as the crop, cardia and copper cell region. Across the gut, *Drosophila* establishes interactions with microbes, which may vary according to its nature. Some microbes interact by processing molecules and influence the availability of nutrients for the host. Microbes also play a role in activating pathways, such as the JNK pathway, which, in turn, activates the JAK/STAT pathway in intestinal stem cells (ISCs), promoting proliferation and gut repair. Between the microbial community, there is an interchange of metabolites, forming a network of interaction and metabolic pathways. Finally, the microbes trigger immunity pathways, such as the immune deficiency (Imd) pathway, which regulates the microbiota in the gut by the release of antimicrobial peptides (AMPs) and reactive oxygen species (ROS).

Drosophila suzukii (Matsumura), or spotted-wing *Drosophila* (SWD)³⁰, although phylogenetically close to *D. melanogaster*, is considered a plant pest, having the special ability to oviposit inside ripening berries (Figure I.2A). Females have a serrated ovipositor, opening a wound in undamaged fruits, from where larvae will hatch and start to feed off, destroying the fruit and ultimately becoming unmarketable³¹. There are several factors influencing oviposition choice by females. The ripening coloration of this pests' main hosts, namely, cherries (*Prunus* sp.), strawberries (*Fragaria x ananassa*), blackberries (*Rubus* sp.), blueberries (*Vaccinium* sp.), raspberries (*Rubus* sp.) and grapes (*Vitis* spp.), may be an attractant factor as all hosts become susceptible in advanced ripeness stages³². Fruits' volatile compounds also influence the egg-laying

choice³³. Other factors, such as intact fruits³⁴, colonization of substrate by some microbes³⁵, and the protein-carbohydrate composition³⁶, contribute to the oviposition choice. Ultimately, a previous report and literature review concluded that raspberries, blackberries and strawberries were the best hosts³⁷.

Although soft-skinned berries are far more likely to attract female flies to infest fruits, alternative non-crop hosts in infested orchards have also been studied and identified. This knowledge lets producers reformulate strategies against SWD according to orchard configuration and surrounding flora^{38–40}. Moreover, overwinter biology is a key aspect when developing strategies against this pest, as they undergo a morphotype change into a winter-acclimated morph, with improved cold-tolerance and reproductive diapause, possibly contributing to the successful invasion and acclimatization of this species^{41–43}. Furthermore, winter morphs were reported to settle in alternative hosts around orchards and cause the first spring infestations of berry orchards after reproductive dormancy⁴⁴.

Similar to other *Drosophila* spp., SWD presents a four-stage life cycle comprising the egg, larva, pupa, and adult, in which the larval stage comprehends 3 developmental instars^{30,45}. The developmental time and success of SWD are, naturally, dependent on several factors, such as diet and temperature. Oviposition marks the start of a new generation, with females reaching a peak of eggs laid at 25°C to 27°C^{46,47}. After hatching from eggs, the individual enters the larval stage. The different instars are distinguished by size, reaching 3 to 4 millimetres at the third instar⁴⁸. It is also very common for wandering third instar larvae to leave the fruit (or artificial medium in laboratory rearing) in search of another pupariation substrate⁴⁹. At optimal temperatures (25°C to 28°C), larvae take between 6 and 7 days to pupariate^{46,47,50} and around 4 days to emerge into adults⁵⁰. Male adult flies present a distinctive feature that facilitates the identification of this species, the presence of a dark spot on the first major wing vein, from which is given “spotted-wing *Drosophila*” name (Figure 1.2B). They also have a pair of tarsal combs sets (also known as sex combs, as they are involved in mating) in each frontal segmented leg oriented parallelly to the length of the leg. Female adults possess a specialized enlarged and serrated ovipositor, as previously mentioned, and do not have tarsal combs nor a dark spot on the wings. Abdomen segments are uninterrupted dark bands at the end of each segment^{51–53}. Winter morphs are distinguished morphologically due to the enhanced melanisation of the abdominal segments and enlarged wings in both sexes^{54,55}.

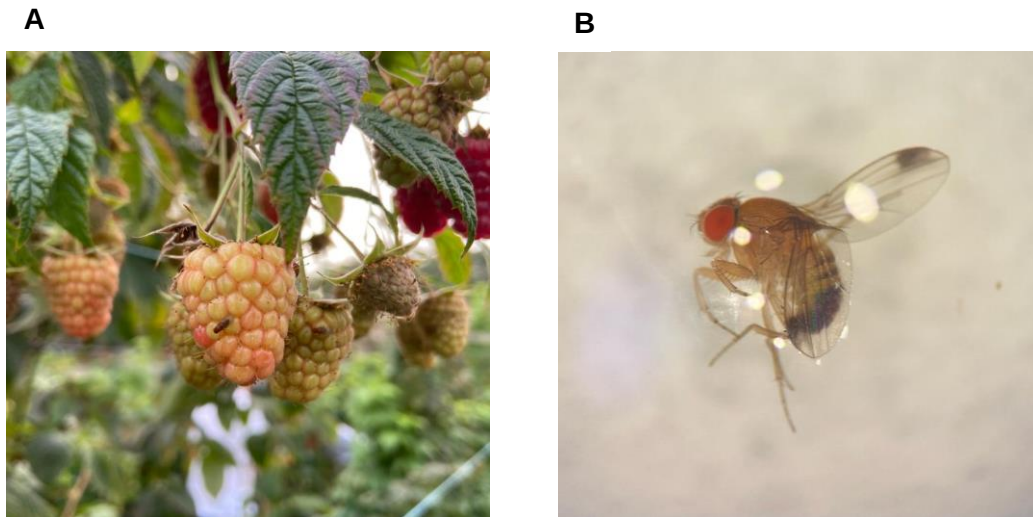


Figure I.2 - *Drosophila suzukii* pest. (A) *D. suzukii* female adults are able to lay eggs inside ripening or ripen red fruits and berries. (B) *D. suzukii* male adults are distinguished for their dark spot in the first vein of both wings.

Nowadays, this pest has an intercontinental distribution and has damaged berry orchards across Europe and America leading to significant economic losses^{56–61} (Figure I.3). A study reveals that moderate temperatures and rainfall are the most suitable areas for SWD establishment, predicting the possible invasion of Australia, New Zealand, and coastal regions of subtropical Africa⁶². This leads to a demand for pest management strategies for this pest to both hinder its spread and control already infested areas.

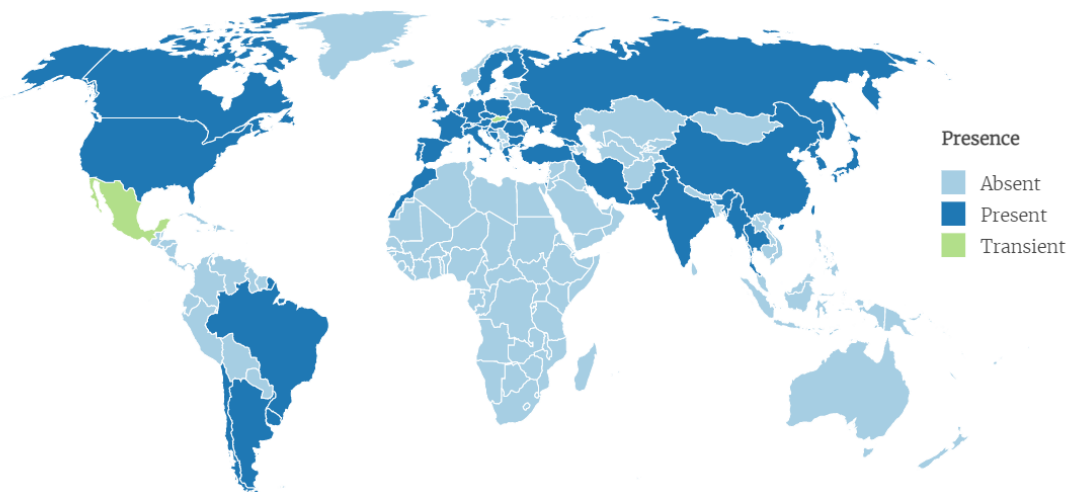


Figure I.3 - Global distribution of *Drosophila suzukii*. Distribution of *D. suzukii* across the world according to the data published by the European and Mediterranean Plant Protection Organization at 26th September 2022: <https://gd.eppo.int/taxon/DROSSU/distribution>.

Similar to other insect pests, SWD's microbiome has been studied over the past decade and progress has been made towards the manipulation of microorganisms as a new approach to pest biological control and the role of these symbionts concerning pest evolution and resistance^{63–65}. This review will dwell on the current state of the SWD microbiome knowledge, particularly the bacteriome, in terms of its composition and function, as well as how this knowledge may help develop new integrated pest control strategies.

2. *Drosophila suzukii* gut bacterial microbiome characterization

The current definition of microbiome and its study does not rely merely on the isolation of microorganisms in laboratory media, as we currently know that only about 1% of microorganisms are able to grow on these artificial conditions⁶⁶. Instead, molecular approaches for identifying the unknown microbial consortium of an ecosystem introduced the concept of metagenomics⁶⁷. This concept has been evolving and the introduction of 16S ribosomal RNA gene for DNA barcoding of bacterial species, and Next-Generation Sequencing for amplicon metagenomics, enhanced the resolution of metagenomics and microbiome characterization. Most of the following studies used these tools to characterize the microbiome of SWD from different sampled regions. Table I-1 summarizes all the genera and species of bacteria identified in SWD for the past years.

In 2014, the first study on the characterization of SWD's microbiome was published⁶⁸. Since then, new taxa have been associated with this species and, similarly to other *Drosophila*, the major representative bacterial families of the gut microbiota of *D. suzukii* are Acetobacteraceae, Enterobacteriaceae, and Lactobacillaceae families^{69–71}. However, there's still a lack of functional and host-microbe interaction characterization in SWD, but the knowledge and similarities with *D. melanogaster* may establish a starting point for the functional characterization of the microbial network in the gut of *D. suzukii*. Another key point when addressing the microbial composition in SWD, as any other understudied organism, is the possible variability. Inter-site and inter-sample variability seems to be a strong influence on microbial variation in *D. suzukii*⁷².

2.1. Acetic Acid Bacteria

The acetic acid bacteria (AAB) are a group of sugar and ethanol oxidizers, Gram-negative bacteria from the Acetobacteraceae family. *D. melanogaster* has previously been shown to, not only, naturally harbour AAB, but to establish physiological interactions important for fitness increase and survival^{71,73,74}. *Drosophila*-associated *Acetobacter* isolates seem to be genetically different from environmental isolates,

which may hint towards key genetic features involved in the host-microbe interaction^{75,76}. Similar to other *Drosophila* species, *D. suzukii* also has been found to harbour different AAB, particularly bacteria from the *Acetobacter*, *Gluconobacter* and *Gluconoacetobacter* genera (see Table I-1)^{68,72,77–80}. However, unlike *D. melanogaster*, very little data is available on the SWD-AAB interaction. In 2017, Vacchini and colleagues revealed a physical interaction between gut-isolated AAB and the peritrophic matrix of the gut of *D. suzukii*, using fluorescence microscopy. Moreover, some species (*Gluconobacter oxydans*, *Acetobacter tropicalis*, and *Acetobacter indonesiensis*) were able to recolonize the gut of the flies after antibiotic treatment⁸¹. This data strongly confirms that SWD is able to associate with AAB, despite the lack of data concerning biochemical and molecular interactions. Interestingly, another study revealed the ability of some SWD-isolated AAB (*Acetobacter malorum*, *Acetobacter persici*, *Gluconobacter frateurii*, and *Gluconobacter japonicus*) to promote *D. melanogaster* larval growth⁸². Not only do these data show an overlapping ecology between the two *Drosophila* species, but it also stresses the importance of AAB in *Drosophila*'s gut. A possible explanation for the ecological significance of AAB in *Drosophila* gut is the ubiquitous presence of AAB in acidic, ethanol and sugar-rich environments such as fruits and the metabolic capacity to incompletely oxidize these compounds into acetic acid⁸³. *Drosophila* gut and diet may provide optimal conditions for AAB metabolism and proliferation, while these bacteria also contribute with certain compounds which improve nutrition and immunity of the flies⁸⁴. *Gluconobacter* genus is particularly able to oxidize glucose into gluconic acid, whose presence correlates with insects associated with a more sugar-rich diet. Although *D. suzukii* oviposits in ripening fruits (sugar-rich), in opposition to rotting fruits (ethanol-rich), *Acetobacter* seems to remain the prevalent genus, confirming that SWD adults are still attracted to fermenting environments and doesn't necessarily feed from the ripening fruits, which has been shown previously⁸⁵. However, identification of bacteria in larvae has shown more prevalence of *Gluconobacter*, which is not surprising as, in this development stage, they can only feed from the sugar-rich diet of ripening fruits^{68,72}.

2.2. Enterobacteriaceae

Enterobacteriaceae is a family of Gram-negative bacteria, known for their presence in animal gut microbiota and vertical heritage, namely in insects, due to their involvement in digestion and detoxification^{86–89}. Not only in *Drosophila* but in other fruit flies (Tephritidae family), Enterobacteriaceae are gut microbiota dominants, as they have been demonstrated to express enzymes that help digestion and improve physiological parameters and amino acid nutrition^{90–93}. However, despite commonly associated with

each other, the interaction between enterobacteria and *Drosophila* remains unclear^{69,71}. The scarce available data reveals that artificially reared *Drosophila* flies, particularly SWD, exhibit a tendency to enrich the proportion of Enterobacteriaceae in the gut, probably due to a diet shift and adaptation, that selectively promote the colonization by these bacteria^{78,80}, whereas the colonization by AAB seems to be promoted by natural fruit-diet⁷⁹. Moreover, a study conducted by Martinez-Sañudo *et al* (2018) revealed an interesting tendency of invasive colonies from different locations (Europe and the United States of America) to harbour a significantly higher proportion of Enterobacteriaceae, in the initial phases of colonization, again suggesting that these bacteria may play a role in an adaptation process and invasion success⁷⁷. Although there is still little to no information at the genus or species level, concerning the functional characterization of these bacteria in the gut of *D. suzukii*, some genera seem to dominate among the Enterobacteriaceae family, such as *Pantoea*, *Dickeya*, *Kluyvera*, and *Lonsdalea*^{77,78,80}, but undoubtedly the *Tatumella* genus stands out in different studies as (one of) the most abundant species, especially in larvae^{68,72,77}. Member species of this genus, some with clinical and pathogenic relevance^{94,95}, have been reclassified from the *Pantoea* genus which can possibly cause some bias in their identification, as they are both closely related^{96,97}. Despite previously identified in *D. melanogaster*⁹⁷, an unidentified species from the *Tatumella* genus isolated from *D. suzukii* was not able to rescue the normal development of axenic *D. melanogaster* larvae, suggesting a specific beneficial interaction with SWD, which has also been previously shown for *Tatumella ptyseos*^{72,82}. Nevertheless, no further studies on the interaction between these two species have been developed. Oviposition might also be influenced by members of this family, namely from the *Enterobacter* genus, which could putatively encode an enzyme required for egg production⁹⁸. This information is a starting point for deepening the knowledge of the role of Enterobacteriaceae species in the gut of insects, which would ultimately be insightful concerning the functional composition of the microbiome.

2.3. Lactic Acid Bacteria

The Lactobacillaceae family, or lactic acid bacteria (LAB), is a group of Gram-positive bacteria belonging to the Firmicutes phylum. The role of LAB in the gut of mammals has been intensively studied, namely in livestock, as they are fermenting bacteria with important industrial applications and probiotic properties^{99–102}. LAB seem to be significantly represented in the gut of *Drosophila* and some species play important roles in fermentative and nucleotide metabolism^{71,103}. One of the most studied species in *Drosophila*, *Lactobacillus plantarum* promotes and coordinates the development and

gut homeostasis of flies, as well as provides anti-oxidative cytoprotection by activation of Cap-n-collar C (CncC, homologous of Nrf2), which elicits the transcription of cytoprotective genes, such as Cytochrome P450 family and Glutathione S-transferases^{71,104–108}. However, interestingly, a study revealed that the monoassociation of *L. plantarum* with *D. melanogaster* slows the regeneration and deteriorates the gut epithelium and longevity¹⁰⁹. Once again, this data reveals the importance of the microbiome as a whole community and not as individual organisms. Another species of LAB, *Lactobacillus rhamnosus* has been associated with pesticide-detoxification, which is an important finding, when studying insect pests¹¹⁰. Despite the significant importance of LAB in *D. melanogaster*, their role in *D. suzukii* is yet to be unveiled. Although some species, such as *Lactobacillus parakefiri*, *Lactobacillus pentosus*, and *Lactobacillus plantarum* have been already isolated from SWD, their importance is not clear. Other LAB, from the *Leuconostoc* genus, have been identified in *D. suzukii* and, in some studies, *Leuconostoc pseudomesenteroides* as dominant species in SWD^{72,103}. However, this species reveals pathogenic activity towards *D. suzukii*^{72,111}, suggesting that possibly this species may establish a relationship with the flies, but also exhibit opportunistic pathogenicity.

2.4. Implications

The identified taxa in the gut of SWD comprise many other groups, from families like Comamonadaceae and genera like *Pseudomonas*, *Bacillus*, *Paenibacillus* and *Staphylococcus*, among others, that also deserve to be further studied on its functional role in the gut of SWD^{72,77–80}. The interaction between the different species is an important factor to take in account as they coexist and establish networks as it's been previously studied for other *Drosophila* models^{24,112,113}.

Given this compilation, much more information is lacking regarding the interactions and molecular cues involved in these host-microbe links. Moreover, the quest to establish a core microbiome needs to be further studied and, as aforementioned, the variability in the results seems to be hindering this process. Another factor to take in account is the fact that most of the available protocols and tools are optimized to other study models, namely *D. melanogaster*, and efforts must be made to develop efficient and standardized protocols for quick identification of microbial symbionts of *D. suzukii*⁸⁰.

The implications on functionally characterizing the microbiome of *D. suzukii* ultimately point to different paths and technological advancements to improve pest management strategies. The first studies on the characterization of its microbiome revealed key aspects that need to be further studied to better understand possible symbiotic and

molecular targets for management. Furthermore, the characterization of the microbiome also may lead to conclusions about pest behaviour, adaptation and success, as the microbiome is known to shape *Drosophila* fitness^{114,115}.

Table I-1 - Bacteria associated with *Drosophila suzukii*. Compilation of species and genera of all bacteria identified in the gut of *D. suzukii* according to different publications, alongside with the method of identification, the developmental stage in which the bacteria were identified, the effect of bacteria when *D. suzukii* is infected with them, the source of *D. suzukii* and the morphotype.

Superkingdom	Phylum	Class	Order	Family	Genus	Species	Method	Development Stage	Infection of <i>D. suzukii</i>	Origin (<i>D. suzukii</i>)	Morphotype	Reference
©Bacteria	Actinobacteria	Actinomycetia	Corynebacteriales	Corynebacteriaceae	<i>Corynebacterium</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
						<i>Corynebacterium glyciniphilum</i>	Isolate	Adults	Detrimental	Wild	Summer	Bing et al (2020)
			Micrococcales	Microbacteriaceae	<i>Microbacterium</i>	<i>Microbacterium foliorum</i>	Isolate	Adults	-	Lab	Summer	Vacchini et al (2017)
					<i>Leucobacter</i>	<i>Leucobacter tardus</i>	Isolate	Adults	Neutral	Wild	Summer	Bing et al (2020)
				Micrococcaceae	<i>Arthrobacter</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
					<i>Kocuria</i>	<i>Kocuria rhizophila</i>	Isolate	Larvae	Beneficial	Wild	Summer	Hiebert et al (2020)
					<i>Micrococcus</i>	-	Isolate	Adults	-	Lab	Summer	Vacchini et al (2017)
						<i>Micrococcus aloeverae</i>	Isolate, Metagenomics	Larvae, Adults	-	Wild	Summer	Hiebert et al (2020), Bing et al (2018)
						<i>Micrococcus yunnanensis</i>	Isolate	Larvae	Beneficial	Wild	Summer	Hiebert et al (2020)
	Bacteroidetes	Cytophagia	Cytophagales	Hymenobacteraceae	<i>Hymenobacter</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
				Spirosomaceae	<i>Dyadobacter</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
		Flavobacteria	Flavobacteriales	Flavobacteriaceae	<i>Flavobacterium</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
				Weeksellaceae	<i>Chryseobacterium</i>	-	Metagenomics	Adults	-	Wild	Summer, Winter	Fountain et al (2014), Martinez-Saáudo et al (2018)
			Sphingobacteriales	Sphingobacteriaceae	<i>Epilithonimonas</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
					<i>Pedobacter</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
					<i>Sphingobacterium</i>	-	Metagenomics	Adults	-	Wild, Lab	Summer, Winter	Lin et al (2021), Fountain et al (2014)
	Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	<i>Bacillus altitudinis</i>	Isolate	Larvae	Detrimental	Wild	Summer	Hiebert et al (2020)
						<i>Bacillus frigiditolerans</i>	Isolate	Larvae	Detrimental	Wild	Summer	Hiebert et al (2020)
						<i>Bacillus pseudomycoides</i>	Isolate	Adults	Neutral	Wild	Summer	Bing et al (2020)
						<i>Bacillus safensis</i>	Isolate	Larvae	Beneficial	Wild	Summer	Hiebert et al (2020)
						<i>Bacillus simplex</i>	Isolate	Larvae	Detrimental	Wild	Summer	Hiebert et al (2020)
						<i>Bacillus wiedmannii</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
						<i>Bacillus zhangzhouensis</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
					<i>Lysinibacillus</i>	<i>Lysinibacillus fusiformis</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
				Paenibacillaceae	<i>Paenibacillus</i>	-	Isolate, Metagenomics	Adults	-	Lab	Summer	Vacchini et al (2017), Lin et al (2021)
						<i>Paenibacillus amylolyticus</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)

						<i>Paenibacillus dongdonensis</i>	Isolate	Larvae	Detrimental	Wild	Summer	Hiebert et al (2020)	
						<i>Paenibacillus odorifer</i>	Isolate	Larvae	Detrimental	Wild	Summer	Hiebert et al (2020)	
						<i>Paenibacillus provencensis</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)	
						<i>Paenibacillus taichungensis</i>	Isolate	Adults	Neutral	Wild	Summer	Bing et al (2020)	
						<i>Paenibacillus xylanexedens</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)	
					Planococcaceae	<i>Kurthia</i>	<i>Kurthia gibsonii</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
					Staphylococcaceae	<i>Staphylococcus</i>	-	Isolate	Adults	-	Lab	Summer	Vacchini et al (2017)
							<i>Staphylococcus capitis</i>	Metagenomics	Adults	-	Wild	Summer	Bing et al (2018)
							<i>Staphylococcus caprae</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
							<i>Staphylococcus epidermidis</i>	Metagenomics	Adults	-	Wild	Summer	Bing et al (2018)
				<i>Staphylococcus haemolyticus</i>			Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)	
				<i>Staphylococcus hominis</i>			Metagenomics	Adults	-	Wild	Summer	Bing et al (2018)	
				<i>Staphylococcus saprophyticus</i>			Isolate	Adults	Detrimental	Wild	Summer	Bing et al (2020)	
				<i>Staphylococcus warneri</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)			
				Lactobacillales	Carnobacteriaceae	<i>Carnobacterium</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
					Enterococcaceae	<i>Enterococcus</i>	<i>Enterococcus faecalis</i>	Metagenomics	Adults	-	Wild	Summer	Bing et al (2018)
					Lactobacillaceae	<i>Lactiplantibacillus</i>	<i>Lactiplantibacillus pentosus</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
							<i>Lactiplantibacillus plantarum</i>	Isolate	Larvae, Pupae	-	Wild, Lab	Summer	Hiebert et al (2020), Vacchini et al (2017)
						<i>Lentilactobacillus</i>	<i>Lentilactobacillus parakefiri</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
						<i>Leuconostoc</i>	-	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)
							<i>Leuconostoc mesenteroides</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
							<i>Leuconostoc pseudomesenteroides</i>	Isolate	Larvae	Detrimental	Wild	Summer	Hiebert et al (2020)
					Streptococcaceae	<i>Lactococcus</i>	<i>Lactococcus lactis</i>	Isolate, Metagenomics	Adults	-	Wild, Lab	Summer	Vacchini et al (2017), Bing et al (2020), Bing et al (2018)

	Proteobacteria	Alphaproteobacteria			<i>Streptococcus</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
						<i>Streptococcus salivarius</i>	Isolate	Adults	-	Lab	Summer	Vacchini et al (2017)
			Caulobacterales	Caulobacteraceae	<i>Brevundimonas</i>	-	Metagenomics	Adults		Wild, Lab	Summer, Winter	Lin et al (2021), Fountain et al (2014)
			Hyphomicrobiales	Methylobacteriaceae	<i>Methylobacterium</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
				Methylocystaceae	<i>Hansschlegelia</i>	<i>Hansschlegelia plantiphila</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
				Rhizobiaceae	<i>Rhizobium</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
			Rhodospirillales	Acetobacteraceae	<i>Acetobacter</i>	<i>Acetobacter cerevisiae</i>	Metagenomics	Larvae, Adults	-	Wild	Summer	Chandler et al (2014)
						<i>Acetobacter cibinongensis</i>	Isolate	Adults	-	Lab	Summer	Vacchini et al (2017)
						<i>Acetobacter indonesiensis</i>	Isolate	Pupae, Adults	-	Lab	Summer	Vacchini et al (2017)
						<i>Acetobacter malorum</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)
						<i>Acetobacter orientalis</i>	Isolate	Adults	-	Lab	Summer	Vacchini et al (2017)
						<i>Acetobacter pasteurianus</i>	Metagenomics	Adults	-	Wild	Summer	Martinez-Sañudo et al (2018)
						<i>Acetobacter peroxydans</i>	Isolate	Adults	-	Lab	Summer	Vacchini et al (2017)
						<i>Acetobacter persici</i>	Isolate, Metagenomics	Pupae, Adults	-	Wild, Lab	Summer	Vacchini et al (2017), Solomon et al (2019), Bing et al (2018)
						<i>Acetobacter tropicalis</i>	Isolate	Adults	-	Lab	Summer	Vacchini et al (2017)
					<i>Asaia</i>	<i>Asaia lannensis</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)
						<i>Asaia siamensis</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)
					<i>Gluconoacetobacter</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
						<i>Gluconoacetobacter liquefaciens</i>	Isolate	Larvae, Pupae	-	Lab	Summer	Vacchini et al (2017)
					<i>Gluconobacter</i>	<i>Gluconobacter cerevisiae</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
						<i>Gluconobacter cerinus</i>	Isolate, Metagenomics	Larvae, Adults	-	Wild	Summer	Hiebert et al (2020), Solomon et al (2019), Chandler et al (2014), Martinez-Sañudo et al (2018)
						<i>Gluconobacter frateurii</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)
						<i>Gluconobacter japonicus</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)
						<i>Gluconobacter kanchanaburiensis</i>	Isolate	Larvae, Pupae, Adults	-	Lab	Summer	Vacchini et al (2017)
						<i>Gluconobacter kondonii</i>	Isolate	Adults	-	Lab	Summer	Vacchini et al (2017)

		Betaproteobacteria				<i>Gluconobacter oxydans</i>	Isolate, Metagenomics	Larvae, Adults	-	Lab, Wild	Summer	Vacchini et al (2017), Chandler et al (2014)
					<i>Komagataeibacter</i>	-	Isolate	Adults	-	Lab	Summer	Vacchini et al (2017)
						<i>Komagataeibacter saccharivorans</i>	Metagenomics	Adults	-	Wild	Summer	Martinez-Sañudo et al (2018)
			Sphingomonadales	Sphingomonadaceae	<i>Sphingomonas</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
						<i>Sphingomonas echinoides</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
						<i>Sphingomonas melonis</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
			Burkholderiales	Alcaligenaceae	<i>Achromobacter</i>	-	Metagenomics	Adults	-	Lab	Summer	Lin et al (2021)
						<i>Achromobacter spanius</i>	Isolate	Adults	Detrimental	Wild	Summer	Bing et al (2020)
					<i>Alcaligenes</i>	-	Metagenomics	Adults	-	Lab	Summer	Lin et al (2021)
						<i>Alcaligenes faecalis</i>	Isolate	Adults	Detrimental	Wild	Summer	Bing et al (2020)
				Burkholderiaceae	<i>Limnobacter</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
				Comamonadaceae	<i>Acidovorax</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
					<i>Comamonas</i>	<i>Comamonas testosteroni</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)
					<i>Delftia</i>	<i>Delftia tsuruhatensis</i>	Isolate	Adults	Neutral	Wild	Summer	Bing et al (2020)
					<i>Lampropedia</i>	-	Metagenomics	Adults	-	Lab	Summer	Lin et al (2021)
				Oxalobacteraceae	<i>Janthinobacterium</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
		Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	<i>Enterobacter</i>	-	Isolate, Metagenomics	Adults	-	Wild	Summer	Solomon et al (2019), Martinez-Sañudo et al (2018), Fountain et al (2014),
						<i>Enterobacter asburiae</i>	Isolate	Adults	Neutral	Wild	Summer	Bing et al (2020)
					<i>Escherichia</i>	<i>Escherichia hermannii</i>	Isolate	Adults	Neutral	Wild	Summer	Bing et al (2020)
					<i>Klebsiella</i>	-	Metagenomics	Adults	-	Lab	Summer	Lin et al (2021)
					<i>Kluyvera</i>	<i>Kluyvera cryocrescens</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)
					<i>Shigella</i>	<i>Shigella boydii</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)
					<i>Siccibacter</i>	-	Metagenomics	Adults	-	Lab	Summer	Lin et al (2021)
					<i>Yokenella</i>	-	Metagenomics	Adults	-	Lab	Summer	Lin et al (2021)
				Erwinaceae	<i>Erwinia</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)
						<i>Erwinia aphidicola</i>	Isolate	Adults	Neutral	Wild	Summer	Solomon et al (2019), Bing et al (2020)

							<i>Erwinia rhapontici</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)	
						<i>Pantoea</i>	<i>Curtobacterium plantarum</i>	Isolate	Larvae	Neutral	Wild	Summer	Hiebert et al (2020)	
							<i>Pantoea agglomerans</i>	Metagenomics	Adults	-	Wild	Summer	Martinez-Sañudo et al (2018)	
							<i>Pantoea brenneri</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)	
							<i>Pantoea vagans</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)	
							<i>Rosenbergiella</i>	<i>Rosenbergiella epipactidis</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)
						<i>Tatumella</i>	-	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)	
							<i>Tatumella ptyseos</i>	Isolate	Larvae	Neutral	Wild	Summer	Hiebert et al (2020)	
							<i>Tatumella punctata</i>	Isolate, Metagenomics	Larvae, Adults	-	Wild	Summer	Hiebert et al (2020), Chandler et al (2014)	
							<i>Tatumella terrea</i>	Isolate	Larvae	Detrimental	Wild	Summer	Hiebert et al (2020)	
						Morganellaceae	<i>Providencia</i>	-	Metagenomics	Adults	-	Lab	Summer	Lin et al (2021)
						Pectobacteriaceae	<i>Dickeya</i>	-	Metagenomics	Adults	-	Wild	Summer	Bing et al (2018)
							<i>Lonsdalea</i>	<i>Lonsdalea querci</i>	Metagenomics	Adults	-	Wild	Summer	Martinez-Sañudo et al (2018)
						Yersiniaceae	<i>Serratia</i>	-	Isolate, Metagenomics	Larvae, Pupae, Adults	-	Lab, Wild	Summer, Winter	Vacchini et al (2017), Fountain et al (2014)
				<i>Serratia marcescens</i>	Isolate			Adults	Detrimental	Wild	Summer	Bing et al (2020)		
				Moraxellales	Moraxellaceae	<i>Acinetobacter</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)	
						<i>Psychrobacter</i>	-	Metagenomics	Adults	-	Wild	Winter	Fountain et al (2014)	
				Oceanospirillales	Halomonadaceae	<i>Candidatus Portiera</i>	-	Metagenomics	Adults	-	Wild	Summer	Bing et al (2018)	
						<i>Zymobacter</i>	<i>Zymobacter palmae</i>	Metagenomics	Adults	-	Wild	Summer	Martinez-Sañudo et al (2018)	
				Orbales	Orbaceae	<i>Orbus</i>	-	Metagenomics	Larvae, Adults	-	Wild	Summer	Chandler et al (2014)	
							<i>Orbus sasakiae</i>	Isolate	Larvae	-	Wild	Summer	Hiebert et al (2020)	
				Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	-	Metagenomics	Adults	-	Lab, Wild	Summer, Winter	Martinez-Sañudo et al (2018), Bing et al (2018), Fountain et al (2014), Lin et al (2021)	
							<i>Pseudomonas coleopterorum</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)	
							<i>Pseudomonas endophytica</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)	
							<i>Pseudomonas geniculata</i>	Isolate	Adults	-	Lab	Summer	Vacchini et al (2017)	
<i>Pseudomonas parafulva</i>	Isolate	Adults	Neutral				Wild	Summer	Bing et al (2020)					

					<i>Pseudomonas putida</i>	Isolate	Adults	-	Wild	Summer	Solomon et al (2019)	
			Xanthomonadales	Rhodanobacteraceae	<i>Dyella</i>	-	Metagenomics	Adults	-	Wild	Summer	Chandler et al (2014)
				Xanthomonadaceae	<i>Stenotrophomonas</i>	-	Metagenomics	Adults		Wild, Lab	Summer, Winter	Fountain et al (2014), Lin et al (2021)

References: Bing *et al* (2018)⁸⁰, Bing *et al* (2021)¹⁵⁵, Chandler *et al* (2014)⁶⁸, Fountain *et al* (2014)²⁰⁶, Hiebert *et al* (2020)⁷², Lin *et al* (2021)⁷⁸, Martinez-Sañudo *et al* (2018)⁷⁷, Solomon *et al* (2019)⁸², Vacchini *et al* (2017)⁸¹

3. Integrated Pest Management (IPM) Strategies for SWD Pest Control

3.1. A synopsis of current control practices

The economic impact of SWD is undeniable and previous reports predicted losses ranging in the order of millions and hundreds of millions across European and American countries, most of them not accounting for pest management costs^{61,116,117}. Cultural practices, such as pruning, short harvesting intervals, removal of infested fruits and net exclusion are important tools to prevent and control the infestation of orchards in an environmentally friendly manner^{118,119}. Nevertheless, large-scale management techniques are required for larger productions. Thus, over the last years, the spotlight shines on the development of alternative strategies, such as mass trapping using synthetic lures, mass-rearing of sterile-induced adult males using radiation, and biological control tools^{120–124}.

Besides cultural practices, the first management tools for this pest relied on the use of effective insecticides^{114,115}. Commercial insecticides may be classified as natural (plant-based or mineral oils), and synthetic^{125–127}. These last ones are divided into several groups according to the chemical structure, and the most commercially important include organochlorines, organophosphates, carbamates, and pyrethroids. They also can be classified according to their cellular targets, and most of the currently marketable insecticides attack the nervous system of insects^{127,128}. This is a highly important classification to elucidate pests' resistance acquisition¹²⁹. Besides targeting the nervous system, insecticides may also target the endocrine system, disrupt the cuticular wax adsorption, the mitochondrial function, or the insect water balance^{129,130}. Among those affecting the nervous system, there are several subclasses also according to the mode of action, including the Acetylcholinesterase disruptors (inhibitors or stimulators) (e.g., carbamates, organophosphates), Gaba-receptor blockers (e.g., organochlorines), Glutamate receptors modulators, and Sodium channel modulators/ blockers (e.g., pyrethroids)^{129,130}.

The hazard risks that these insecticides pose (e.g., in terms of environmental impact on beneficial insects and bioaccumulation, or in terms of food safety), raised the need of developing and adopting green IPM strategies. An example of this consciousness is the European Union (EU) Farm to Fork strategy, towards substantially reducing the use of pesticides, including insecticides, in the next years¹³¹. This regulation imposes the development of new alternative strategies, avoiding for example the undesirable toxicity to aquatic life, non-target insects, namely pollinators, and natural predators or

parasitoids, and avoiding the accumulation of the insecticide in the fruits, prior their harvest and transfer to the market^{132–137}. Moreover, the highly polyphagous nature, high fertilization rate, number of offspring, and morphological adaptation of *D. suzukii* can decrease the effectiveness of chemical control strategies, leading to frequent outbreaks in red fruit-producing regions¹³⁸. This has created enormous pressure on producers to intensively apply insecticides against this pest. This continuous use, besides being a risk to beneficial insects, was also shown to induce resistant populations in the field as reported already for laboratory and natural populations with increased resistance^{139–145}.

Thirdly, the biological control tools for SWD, currently, comprise different approaches, namely parasitoids, predators, and entomopathogens (bioinsecticides), depicted in Figure I.4. Briefly, parasitoids take advantage of the host (mostly in the larval or pupal phase) killing the host. Different species of parasitoids have shown promising biological control towards SWD, for instance, the insects *Trichopria drosophilae* and *Asobara japonica*¹²⁴. That said, the release of these parasitoids must be carefully studied for ecological hazards as the introduction of non-native species could lead to ecological disruption, due to the variable host specificity of these insects. In this regard, Girord *et al* (2018), assessed the host specificity of different parasitoids and found that even the most specific species *Ganaspis cf. brasiliensis* revealed variation according to origin¹⁴⁶.

Predators of SWD have been identified by exposing larvae, pupae, or adults to predators and by PCR identification in the field. Organisms from Araneae, Coleoptera, Dermaptera, Hemiptera, and Orthoptera have been proved predate on SWD^{124,147}. Recently, we have demonstrated that also Opiliones and Hemerobiidae fed on *D. suzukii* in the field¹⁴⁷. However, although several predators have been tested in an artificial environment, they have yet to be tested in a natural/field context for their efficacy and safety. Plus, a key limitation to a broader use of predators is that they usually have a broad spectrum of feeding taxa, being not specific for this pest, but also feeding on beneficial species.

Using entomopathogens to control this pest is another approach with growing interest. Naturally, entomopathogens are important regulatory factors in insect populations and plant protectors¹⁴⁸. Some of those entomopathogens are already used in biological control, being examples of how introducing a pathogen in the pest (and its microbiome) may be a powerful tool to control this pest^{149,150}. In the following section we'll address the importance of entomopathogens and other approaches to manipulate pests' microbiomes.

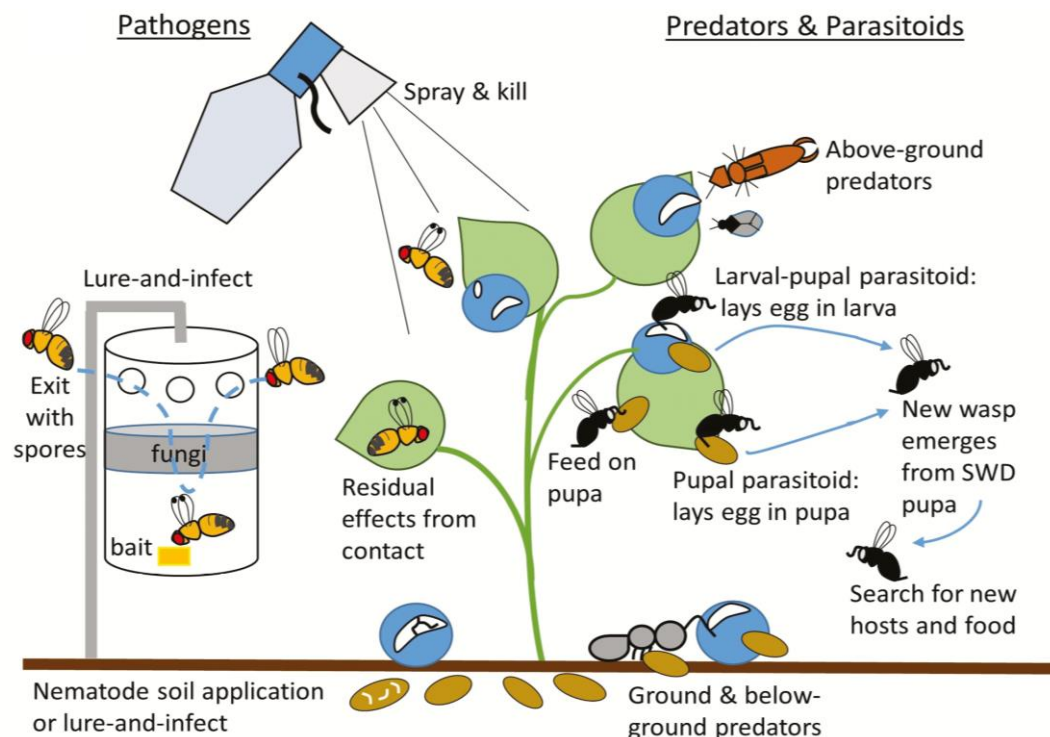


Figure I.4 - Biological control methods of the *Drosophila suzukii* pest. Strategies of biological control comprising different agents, such as predators, parasitoids, entomopathogenic nematodes and microorganisms (fungi and bacteria). These agents may be applied according to different methods and act upon different developmental stages. Figure adapted from Lee et al (2019)¹⁵¹ (authorized).

3.2. Manipulating microbiomes as an emerging control strategy

At a specific time, the SWD microbiome integrates its inner and outer communities of core (mostly symbionts) or transient microorganisms. These may interact with the host negatively (e.g., entomopathogens), neutrally or positively. One may consider fungi, bacteria and, in parallel, the importance of the SWD-virome is also emerging.

3.2.1. Entomopathogens

Some entomopathogenic bacteria are already commercially used as insecticides, for instance, *Bacillus thuringiensis*, a widely studied and tested entomopathogen^{152,153}. Regarding, SWD, different generic bacterial and fungal entomopathogens have been tested against this pest. Studies conducted with variants of *Bacillus thuringiensis*, *Beauveria bassiana*, *Isaria fumosorosea*, and *Metarhizium anisopliae* all have shown detrimental activity and increased mortality in exposed SWD¹²⁴. However, similar questions to parasitoids and predators' applicability, rise here as to the specificity for non-target insects, or the safety of introducing different microbial species into the environment¹⁵⁴.

To assess this issue, the study of biological control agents of SWD turned to the identification of natural pathogens and symbionts of this species, aiming to overcome

the issue of the introduction of new microbial species. Studies are now assessing the pathogenic activity of naturally-isolated bacteria from wild-caught flies^{72,155}.

Currently, the majority of the studies of entomopathogenic bacteria against pests rely on generic entomopathogens and mass production for general bioinsecticide application^{156–158}. Although these are more environmentally friendly approaches than the widely used pesticides, the establishment of pest-specific control agents would reduce indirect costs in the ecological parameters. In search of new IPM strategies, two recent studies aimed to isolate natural pathogens of SWD^{72,155}. These screenings of new entomopathogens of this pest open new doors for effective and less environmental invasive tools, and show how manipulating entomopathogenic bacteria may succeed in controlling the pest.

In the study conducted by Bing *et al* (2020), they established a consortium of wild-SWD-isolated bacteria (Table I-1) and studied their pathogenic potential against SWD by performing survival assays, which comprise septic and oral infections. These assays revealed that septic infection of SWD with *Achromobacter spanius*, *Alcaligenes faecalis*, *Serratia marcescens*, *Corynebacterium glycinophilum*, *Lactococcus lactis*, and *Staphylococcus saprophyticus* had a detrimental activity towards the fly, ranging from total mortality to decreased survival, whereas the oral infection showed no significant effect with any bacteria at a lower concentration but when increased the feeding concentration, *Ac. spanius* exhibited pathogenic activity towards SWD. These results suggest that although some of these bacteria might cause systemic infection in the organism when entering the hemolymph, they do not necessarily exert any detrimental activity when ingested (possibly being opportunistic pathogens), which would ultimately be the goal for spray-application of bioinsecticides. To complete this assessment, they hypothesized that oral infection could have different outcomes in adults vs larvae, so they performed survival assays for SWD larvae with *Ac. Spanius*, *Al. faecalis* and *S. marcescens*, all showing a significant increase in larvae mortality, proving that pathogenic activity may be stage-dependent, opening new insights into the targeting of bioinsecticides. Despite showing interesting results, most pathogenic bacteria pose health risks for humans as they cause infections or have been previously isolated from clinical samples^{159–162}. Regardless, the authors showed a proof of concept as natural pathogens of SWD may be isolated from wild flies, opening new doors to more robust studies and screening of natural pathogens, and eventually use them in the pest's control.

In the same year, Hiebert *et al* (2020) conducted a study, previously mentioned, where they isolated microorganisms from SWD moribund larvae. *L. pseudomesenteroides* and *Tatumella ptyseos* were the most abundant species in the obtained consortia. Following this characterization, similarly to Bing *et al* (2020), they also aimed to identify natural entomopathogens of SWD. Thus, they performed survival assays by feeding some of the isolated bacteria and identified 7 detrimental microorganisms: *Brevibacterium frigoritolerans*, *Bacillus simplex*, *Bacillus altitudinis*, *Leuconostoc pseudomesenteroides*, *Paenibacillus dongdonensis*, *Tatumella terreus* and *Paenibacillus odorifer*. They also observed that *L. pseudomesenteroides* most likely exerts its detrimental activity by hindering food uptake in the flies. Moreover, they also identified beneficial 3 species of bacteria that increased survival: *Kocuria rhizophila*, *Bacillus safensis* and *Micrococcus yunnanensis*⁷².

To further complement this assessment, the authors aimed to increase the complexity of interaction, based on the premise that flies would not likely host only a single species of bacteria at a given time. They, then, performed a mix of 2 bacteria (1 detrimental mixed with 1 beneficial) on variable proportions ranging from 100:0 to 0:100, and found that in 4 pairs (*K. rhizophila* and *B. frigoritolerans*, *K. rhizophila* and *L. pseudomesenteroides*, *M. yunnanensis* and *B. frigoritolerans*, and *M. yunnanensis* and *L. pseudomesenteroides*), there seems to be a proportion-dependent correlation with survival in comparison to control. Although, this assay was not able to distinguish if increasing in survival with lower concentrations of detrimental bacteria was due to (1) less concentration of detrimental bacteria or (2) to an antagonistic effect of the increasing concentration of beneficial bacteria that enabled to overcome the detrimental activity. Finally, they assessed this issue at a molecular level by evaluating gene expression of some immunity genes but no strong correlation was found, even though the assessed genes (Toll pathway genes and Drosomycin-like peptides) have been previously linked to infections and microbiota in *Drosophila*^{72,163,164}.

These studies provided the first overview of the interaction between host and natural microbes and towards the identification of new biocontrol tools and targets. To further explore these host-microorganism interactions, molecular techniques may be applied and new gene targets may be used to assess the molecular changes in the presence of potential beneficial and detrimental bacteria^{19,165}.

3.2.2. *Drosophila* immunity

Immunity has been thoroughly studied in *D. melanogaster* as a model for human immunity translational studies¹⁶⁶. The homology of immune-related responses at the

organ, cellular and molecular levels has turned this species into a valuable model to study human immunity¹⁶⁷. Particularly, the microbial recognition and eliciting of molecular response pathways has been conserved throughout evolution and the metazoan realm¹⁶⁸. The immune deficiency (Imd) and Toll signalling pathways are of great importance in the humoral immune response, which comprehends the molecular processes starting at the recognition of microbial patterns up until the release of antimicrobial peptides (AMPs)¹⁶⁹. Figure I.5 depicts a schematization of the Imd and Toll pathways from the receptors until the targets.

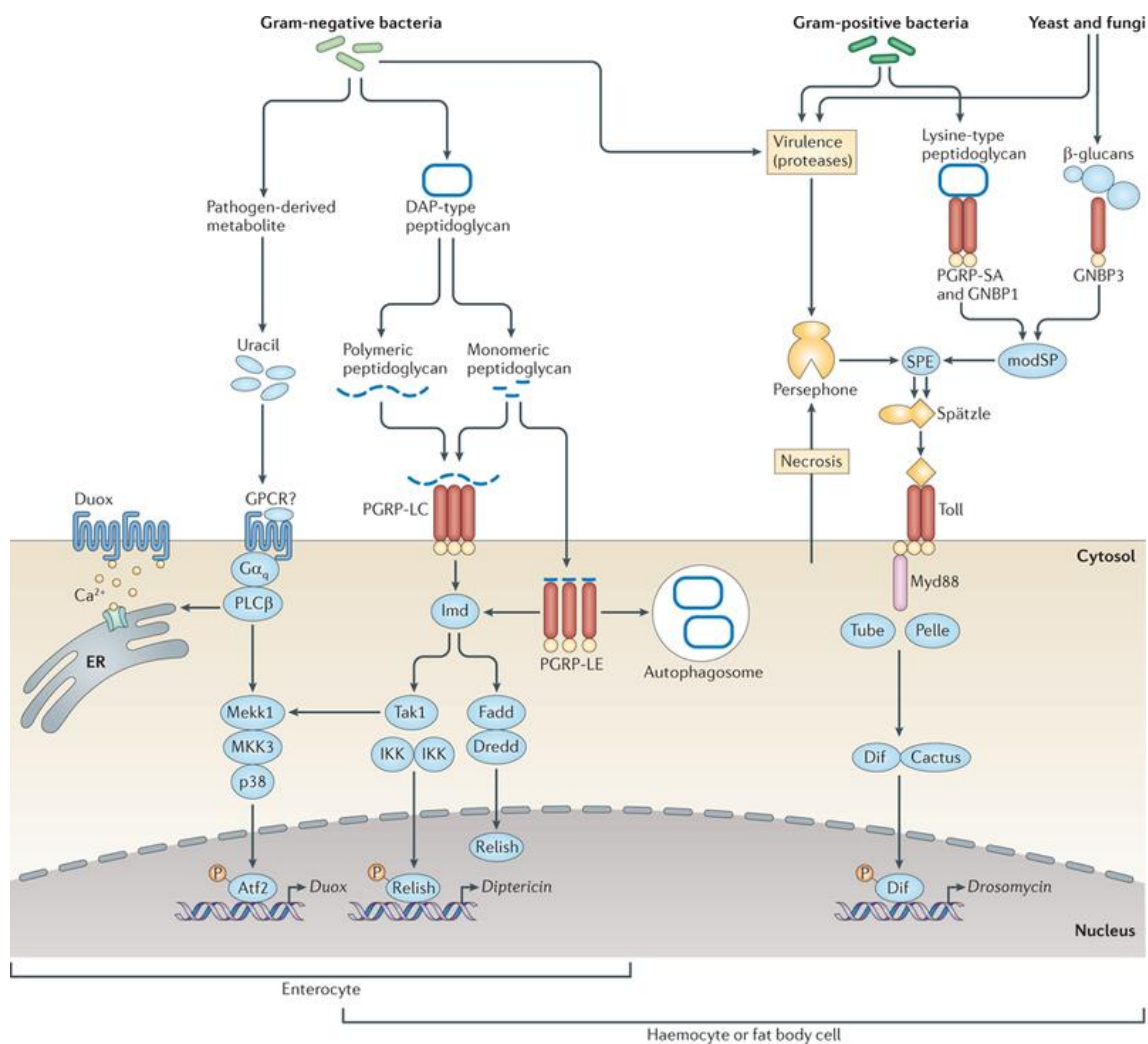


Figure I.5 - The Imd and Toll pathways in *Drosophila*. The Toll pathway is activated mainly by Gram-positive bacteria and fungi. The peptidoglycan receptor proteins, PGRP-SA, GNBP1 and GNBP3 are responsible for the activation of the modular serine protease (modSP) that activate the Spätzle-processing enzyme (SPE), which, in turn, cleaves Spätzle. Spätzle is a ligand of the Toll receptor, eliciting a signalling response in the cell, via the activation of Myd88, Tube, Pelle and Dif, a transcription factor, responsible for the expression of Drosomycin. The Imd pathway is mainly activated by Gram-negative bacteria, recognized by PGRP-LC and PGRP-LE, starting a signalling cascade by activating Imd, which, in turn, activates Tak1 and IKK, enabling the translocation of phosphorylated Relish, a transcription factor, to the nucleus and activating the expression of Diptericin. Another ramification of the Imd pathway leads to the activation of Mekk1. However, the activation of this MAPKKK can be accomplished independently of Imd activation, by the recognition of pathogen-derived metabolites. Mekk1 will then lead to the activation of the MAPK p38, culminating in the transcription of the Dual-oxidase by the Atf2. Figure retrieved from Buchon *et al* (2018)¹⁶⁷ (authorized).

The Imd pathway is involved in a rapid response to bacterial infection, while the Toll pathway is associated with a delayed and persistent response^{170,171}. Imd is ubiquitously expressed in *Drosophila* tissues and requires the recognition of peptidoglycans by peptidoglycan recognition proteins (PGRPs), which can be secreted, transmembrane or cytoplasmic and present receptor or amidase activity¹⁷². PGRP-LC is the transmembrane main elicitor of the Imd pathway in the gut¹⁷³. Together with PGRP-LE, a cytoplasmic receptor, they both recognize, mainly, Gram-negative bacteria and activate Imd¹⁷⁴. The signalling cascade undergoes a process of phosphorylations and peptide cleavages of the signalling components, Imd, Tak1, IKK, Dredd and the NF- κ B protein Relish, a transcription factor (TF), which translocates to the nucleus and activates AMP expression (e.g., Diptericin)¹⁷⁵. Negative regulators of the Imd pathway present in the form of PGRPs (e.g., PGRP-LB, PGRP-S1A, PGRP-SC1B, PGRP-SC2)^{172,176,177}, target genes, such as, Pirk¹⁷⁸ and a plethora of regulatory ubiquitination and SUMOylation processes¹⁷².

The Imd pathway branches into other signalling pathways, namely, the expression of the Dual-oxidase (Duox), which is involved in the regulation of the gut microbiota by the release of reactive oxygen species¹⁷⁹. This enzyme is expressed in an Imd-dependent and independent way¹⁸⁰. Unlike the other pathways, a G-protein-coupled receptor is activated through the coupling of uracil metabolites secreted by bacteria in the gut^{181,182}. Posterior activation of a mitogen-activated protein kinase kinase kinase (MAPKKK), Mekk1, is a convergence point with the Imd pathway as it is also regulated by Tak1¹⁸³. Subsequent activation of the MAPK p38 is responsible for the activation of the Atf2 TF which, in turn, activates the expression of Duox¹⁸⁰.

The Toll pathway is another important immune signalling pathway associated with the response to Gram-positive bacterial and fungal infections¹⁸⁴. The recognition of microorganisms is carried mainly by PGRP-SA, GGBP1 and β -glucans receptor GGBP3, which activate the modular serine protease^{185,186}. Toll receptor activation is preceded by the cleavage of Spätzle by the Spätzle-processing enzyme and culminates in the activation of the NF- κ B proteins Dorsal and Dorsal-related immunity factor (Dif), also through a series of phosphorylation and cleavage events of MyD88, Tube, Pelle, Traf2 and Cactus¹⁷⁵. Dorsal is a TF associated with the dorsoventral patterning of *Drosophila* embryos¹⁸⁷. In turn, although related to Dorsal, Dif does not participate in embryo development and strictly associates with immune response¹⁸⁸. The activation of these TF will then upregulate the expression of AMPs (e.g., Drosomycin)¹⁸⁹.

Although this characterization was built for *D. melanogaster*, SWD presents homolog genetic structures, which facilitates the study of these pathways. However, even though there are some studies on immunity combat upon infection^{190,191}, there is still a lack of studies in expression of the Imd and Toll pathways and their involvement in the combat of entomopathogenic bacteria in SWD. Gene expression analysis has previously shown that the response of SWD to stress stimuli differs from *D. melanogaster*, raising even more the importance of studies on this subject to obtain detailed studies and characterization of this pest's biology and molecular response to new putative natural entomopathogens^{192,193}.

3.2.3. Disrupting the microbiome homeostasis

Considering that normal microbiota (in particular, the bacterial that is the focus of this thesis), influences the host survival, performance and behaviours, disrupting this balance may increase the death rate of the host's population¹⁹⁴. A main target is the influence in the host's immunity¹⁹⁵. Other diseases, nutritional disorders, imbalance of gut repair, reduced stress tolerance and other infections by opportunistic pathogens may occur with the disruption of the gut homeostasis^{196–199}. Several variables can disturb this balance, like malnutrition or infections^{200,201}. The impact of the microbiome to modulate the host homeostasis and disease is well known in *D. melanogaster*, but less in SWD^{20,202}. *D. melanogaster* core microbiome integrates usually species from the *Lactobacillus* and *Acetobacter* genera, among others, being many in symbiotic relations, and thus disrupting this symbiotic relation may also work on weakening the host^{20,21,69,203,204}. For example, it is well demonstrated that axenic individuals are fragile, and do not reproduce in a sustainable way²⁰⁵. Thus, disrupting this homeostasis may be one pivotal strategy to control pests like SWD.

4. Final Remarks

In response to green deals, such as the European Green Deal, new tools are needed to achieve better ecological-oriented agricultural practices. Scientific advances have been developed towards this goal and the manipulation of the microbiome of insects, using natural pathogens, emerged as a new promising cutting-edge technology. The SWD pest, after invading most of Europe and North America, has since threatened the economic stability of soft-skinned red fruits and berries producers. Its repercussions led to an increased interest regarding its biology, in the last decade. For the past 8 years, characterizing its microbiome has also been part of this journey and challenge due to the significant variability between locations, which ultimately impairs the establishment of a core microbiome. Nevertheless, some families of bacteria seem to be related to

SWD, despite studies on the functional characterization on the gut of the flies. Applying the aforementioned concepts, recent publications show the first steps and proof-of-concept on the identification of natural pathogenic bacteria towards SWD, which opens doors to a whole spectrum of environmentally friendly strategies: use of natural cohabiting species of entomopathogenic bacteria against the pest, genetic manipulation of new naturally occurring species of bacteria and screening of new bacterial symbiotic targets.

References

1. Bosch, T. C. G. & McFall-Ngai, M. J. Metaorganisms as the new frontier. *Zoology (Jena)* **114**, 185 (2011).
2. Clemmons, B. A., Voy, B. H. & Myer, P. R. Altering the Gut Microbiome of Cattle: Considerations of Host-Microbiome Interactions for Persistent Microbiome Manipulation. *Microb Ecol* **77**, 523–536 (2019).
3. Weiland-Bräuer, N. *et al.* The Native Microbiome is Crucial for Offspring Generation and Fitness of *Aurelia aurita*. *mBio* **11**, 1–20 (2020).
4. Chan, J., Geng, D., Pan, B., Zhang, Q. & Xu, Q. Gut Microbial Divergence Between Three Hadal Amphipod Species from the Isolated Hadal Trenches. *Microb Ecol* **84**, 1–11 (2021).
5. Xie, K. *et al.* Dietary *S. maltophilia* induces supersized lipid droplets by enhancing lipogenesis and ER-LD contacts in *C. elegans*. *Gut Microbes* **14**, 2013762 (2022).
6. Zhang, S. *et al.* Comparative metabolomic analysis reveals shared and unique chemical interactions in sponge holobionts. *Microbiome* **10**, 1–14 (2021).
7. Turnbaugh, P. J. *et al.* The Human Microbiome Project. *Nature* **449**, 804–810 (2007).
8. Medina, V. *et al.* Characterized non-transient microbiota from stinkbug (*Nezara viridula*) midgut deactivates soybean chemical defenses. *PLoS One* **13**, e0200161 (2018).
9. Ramalho, M. O., Bueno, O. C. & Moreau, C. S. Species-specific signatures of the microbiome from *Camponotus* and *Colobopsis* ants across developmental stages. *PLoS One* **12**, e0187461 (2017).
10. Dillon, R. J., Webster, G., Weightman, A. J. & Keith Charnley, A. Diversity of gut microbiota increases with aging and starvation in the desert locust. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* **97**, 69–77 (2010).
11. Spor, A., Koren, O. & Ley, R. Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat Rev Microbiol* **9**, 279–290 (2011).
12. Furman, O. *et al.* Stochasticity constrained by deterministic effects of diet and age drive rumen microbiome assembly dynamics. *Nat Commun* **11**, 1–13 (2020).
13. Jing, T. Z., Qi, F. H. & Wang, Z. Y. Most dominant roles of insect gut bacteria: digestion, detoxification, or essential nutrient provision? *Microbiome* **8**, 1–20 (2020).

14. Douglas, A. E. Multiorganismal Insects: Diversity and Function of Resident Microorganisms. *Annu Rev Entomol* **60**, 17–34 (2015).
15. Dillon, R. J. & Dillon, V. M. THE GUT BACTERIA OF INSECTS: Nonpathogenic Interactions. *Annu Rev Entomol* **49**, 71–92 (2003).
16. Hosokawa, T., Kikuchi, Y., Shimada, M. & Fukatsu, T. Obligate symbiont involved in pest status of host insect. *Proceedings of the Royal Society B: Biological Sciences* **274**, 1979–1984 (2007).
17. Moran, N. A. & Yun, Y. Experimental replacement of an obligate insect symbiont. *Proc Natl Acad Sci U S A* **112**, 2093–2096 (2015).
18. Vacheron, J. *et al.* T6SS contributes to gut microbiome invasion and killing of an herbivorous pest insect by plant-beneficial *Pseudomonas protegens*. *The ISME Journal* **2019 13:5** **13**, 1318–1329 (2019).
19. Trinder, M., Daisley, B. A., Dube, J. S. & Reid, G. *Drosophila melanogaster* as a high-throughput model for host-microbiota interactions. *Front Microbiol* **8**, 751 (2017).
20. Schönborn, J. W. *et al.* Modeling *Drosophila* gut microbe interactions reveals metabolic interconnectivity. *iScience* **24**, 103216 (2021).
21. Mazzucco, R. & Schlötterer, C. Long-term gut microbiome dynamics in *Drosophila melanogaster* reveal environment-specific associations between bacterial taxa at the family level. *Proceedings of the Royal Society B: Biological Sciences* **288**, 20212193 (2021).
22. Shin, S. C. *et al.* *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science* (1979) **334**, 670–674 (2011).
23. Brown, J. B. *et al.* An integrated host-microbiome response to atrazine exposure mediates toxicity in *Drosophila*. *Commun Biol* **4**, 1–12 (2021).
24. McMullen, J. G., Peters-Schulze, G., Cai, J., Patterson, A. D. & Douglas, A. E. How gut microbiome interactions affect nutritional traits of *Drosophila melanogaster*. *J Exp Biol* **223**, jeb227843 (2020).
25. Henry, Y. & Colinet, H. Microbiota disruption leads to reduced cold tolerance in *Drosophila* flies. *Science of Nature* **105**, 1–5 (2018).
26. Lee, K. A. *et al.* Bacterial-Derived Uracil as a Modulator of Mucosal Immunity and Gut-Microbe Homeostasis in *Drosophila*. *Cell* **153**, 797–811 (2013).

27. Schretter, C. E. *et al.* A gut microbial factor modulates locomotor behavior in *Drosophila*. *Nature* **563**, 402 (2018).
28. Jia, Y. *et al.* Gut microbiome modulates *Drosophila* aggression through octopamine signaling. *Nat Commun* **12**, 1–12 (2021).
29. Hang, S. *et al.* The acetate switch of an intestinal pathogen disrupts host insulin signaling and lipid metabolism. *Cell Host Microbe* **16**, 592 (2014).
30. Kanzawa, T. Studies on *Drosophila suzukii* Mats. *Review of Applied Entomology* **29**, 622 (1939).
31. Lee, J. C. *et al.* In Focus: Spotted wing *Drosophila*, *Drosophila suzukii*, across perspectives. *Pest Manag Sci* **67**, 1349–1351 (2011).
32. Cai, P. *et al.* Potential host fruits for *Drosophila suzukii*: olfactory and oviposition preferences and suitability for development. *Entomol Exp Appl* **167**, 880–890 (2019).
33. Lee, J. C. *et al.* The susceptibility of small fruits and cherries to the spotted-wing *Drosophila*, *Drosophila suzukii*. *Pest Manag Sci* **67**, 1358–1367 (2011).
34. Kienzle, R. & Rohlf, M. Mind the wound!—fruit injury ranks higher than, and interacts with, heterospecific cues for *Drosophila suzukii* oviposition. *Insects* **12**, 424 (2021).
35. Sato, A., Tanaka, K. M., Yew, J. Y. & Takahashi, A. *Drosophila suzukii* avoidance of microbes in oviposition choice. *R Soc Open Sci* **8**, (2021).
36. Silva-Soares, N. F., Nogueira-Alves, A., Beldade, P. & Mirth, C. K. Adaptation to new nutritional environments: Larval performance, foraging decisions, and adult oviposition choices in *Drosophila suzukii*. *BMC Ecol* **17**, 1–13 (2017).
37. Olazcuaga, L. *et al.* Oviposition Preference and Larval Performance of *Drosophila suzukii* (Diptera: Drosophilidae), Spotted-Wing *Drosophila*: Effects of Fruit Identity and Composition. *Environ Entomol* **48**, 867–881 (2019).
38. Lee, J. C. *et al.* Infestation of Wild and Ornamental Noncrop Fruits by *Drosophila suzukii* (Diptera: Drosophilidae). *Ann Entomol Soc Am* **108**, 117–129 (2015).
39. Grant, J. A. & Sial, A. A. Evaluation of Wild Flora Surrounding Blueberry Fields as Viable Hosts of *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae) in Georgia. *Insects* **12**, 667 (2021).
40. Klick, J. *et al.* Distribution and activity of *Drosophila suzukii* in cultivated raspberry and surrounding vegetation. *Journal of Applied Entomology* **140**, 37–46 (2016).

41. Toxopeus, J., Jakobs, R., Ferguson, L. v., Gariepy, T. D. & Sinclair, B. J. Reproductive arrest and stress resistance in winter-acclimated *Drosophila suzukii*. *J Insect Physiol* **89**, 37–51 (2016).
42. Zerulla, F. N., Schmidt, S., Streitberger, M., Zebitz, C. P. W. & Zelger, R. On the overwintering ability of *Drosophila suzukii* in South Tyrol. *J Berry Res* **5**, 41–48 (2015).
43. Stephens, A. R., Asplen, M. K., Hutchison, W. D. & Venette, A. R. C. Cold Hardiness of Winter-Acclimated *Drosophila suzukii* (Diptera: Drosophilidae) Adults. *Environ. Entomol* **44**, 1619–1626 (2015).
44. Panel, A. D. C. *et al.* Overwintered *Drosophila suzukii* Are the Main Source for Infestations of the First Fruit Crops of the Season. *Insects* **9**, (2018).
45. Kaneshiro KY. *Drosophila* (Sophophora) *suzukii* (Matsumura). *Proc Hawaiian Entomol Soc* **24**, 179 (1983).
46. Winkler, A., Jung, J., Kleinhenz, B. & Racca, P. Estimating temperature effects on *Drosophila suzukii* life cycle parameters. *Agric For Entomol* **23**, 361–377 (2021).
47. Kinjo, H., Kunimi, Y. & Nakai, M. Effects of temperature on the reproduction and development of *Drosophila suzukii* (Diptera: Drosophilidae). *Appl Entomol Zool* **49**, 297–304 (2014).
48. van Timmeren, S., Diepenbrock, L. M., Bertone, M. A., Burrack, H. J. & Isaacs, R. A Filter Method for Improved Monitoring of *Drosophila suzukii* (Diptera: Drosophilidae) Larvae in Fruit. *J Integr Pest Manag* **8**, 23 (2017).
49. Hamby, K. A. *et al.* Biotic and abiotic factors impacting development, behavior, phenology, and reproductive biology of *Drosophila suzukii*. *J Pest Sci (2004)* **89**, 605–619 (2016).
50. Tochen, S. *et al.* Temperature-related development and population parameters for *Drosophila suzukii* (Diptera: Drosophilidae) on cherry and blueberry. *Environ Entomol* **43**, 501–510 (2014).
51. Vlach, J. Identifying *Drosophila suzukii* Oregon Department of Agriculture. (2013).
52. Hauser, M. A historic account of the invasion of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) in the continental United States, with remarks on their identification. *Pest Manag Sci* **67**, 1352–1357 (2011).

53. Cinil', A., Ioriatti¹, C. & Anfora¹, G. A review of the invasion of *Drosophila suzukii* in Europe and a draft research agenda for integrated pest management. *Bull Insectology* **65**, 149–160 (2012).
54. Shearer, P. W. *et al.* Seasonal cues induce phenotypic plasticity of *Drosophila suzukii* to enhance winter survival. *BMC Ecol* **16**, 1–18 (2016).
55. Tran, A. K., Hutchison, W. D. & Asplen, M. K. Morphometric criteria to differentiate *Drosophila suzukii* (Diptera: Drosophilidae) seasonal morphs. *PLoS One* **15**, e0228780 (2020).
56. Dean, D., Price, J. F., Steck, G. & Nagle, C. A. Development and Impact of the Spotted Wing *Drosophila*, *Drosophila suzukii*, in Florida Strawberries. *International Journal of Fruit Science* **13**, 67–75 (2013).
57. de Ros, G., Conci, S., Pantezzi, T. & Savini, G. The economic impact of invasive pest *Drosophila suzukii* on berry production in the Province of Trento, Italy. *J Berry Res* **5**, 89–96 (2015).
58. Farnsworth, D. *et al.* Economic analysis of revenue losses and control costs associated with the spotted wing *Drosophila*, *Drosophila suzukii* (Matsumura), in the California raspberry industry. *Pest Manag Sci* **73**, 1083–1090 (2017).
59. Knapp, L., Mazzi, D. & Finger, R. The economic impact of *Drosophila suzukii*: perceived costs and revenue losses of Swiss cherry, plum and grape growers. *Pest Manag Sci* **77**, 978 (2021).
60. de Ros, G., Grassi, A. & Pantezzi, T. Recent Trends in the Economic Impact of *Drosophila suzukii*. *Drosophila suzukii Management* 11–27 (2020).
61. de Ros, G., Anfora, G., Grassi, A. & Ioriatti, C. The potential economic impact of *Drosophila suzukii* on small fruits production in Trentino (Italy). *Integrated protection of fruit crops IOBC-WPRS Bulletin* **91**, 317–321 (2013).
62. Santos, L. A. dos *et al.* Global potential distribution of *Drosophila suzukii* (Diptera, Drosophilidae). *PLoS One* **12**, 1–13 (2017).
63. Agarwal, A. & Agashe, D. The red flour beetle *Tribolium castaneum*: A model for host-microbiome interactions. *PLoS One* **15**, e0239051 (2020).
64. Boush, M. G. & Matsumura, F. Insecticidal Degradation by *Pseudomonas melophthora*, the Bacterial Symbiote of the Apple Maggot. *J Econ Entomol* **60**, 918–920 (1967).

65. Ceja-Navarro, J. A. *et al.* Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. *Nat Commun* **6**, 1–9 (2015).
66. Kaeberlein, T., Lewis, K. & Epstein, S. S. Isolating ‘uncultivable’ microorganisms in pure culture in a simulated natural environment. *Science* (1979) **296**, 1127–1129 (2002).
67. Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J. & Goodman, R. M. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem Biol* **5**, R245–R249 (1998).
68. Chandler, J. A., James, P. M., Jospin, G. & Lang, J. M. The bacterial communities of *Drosophila suzukii* collected from undamaged cherries. *PeerJ* **2**, e474 (2014).
69. Broderick, N. A. & Lemaitre, B. Gut-associated microbes of *Drosophila melanogaster*. *Gut Microbes* **3**, 307–321 (2012).
70. Staubach, F., Baines, J. F., Künzel, S., Bik, E. M. & Petrov, D. A. Host Species and Environmental Effects on Bacterial Communities Associated with *Drosophila* in the Laboratory and in the Natural Environment. *PLoS One* **8**, (2013).
71. Chandler, J. A., Lang, J., Bhatnagar, S., Eisen, J. A. & Kopp, A. Bacterial communities of diverse *Drosophila* species: Ecological context of a host-microbe model system. *PLoS Genet* **7**, (2011).
72. Hiebert, N., Carrau, T., Bartling, M., Vilcinskas, A. & Lee, K. Z. Identification of entomopathogenic bacteria associated with the invasive pest *Drosophila suzukii* in infested areas of Germany. *J Invertebr Pathol* **173**, 1–11 (2020).
73. Corby-Harris, V. *et al.* Geographical distribution and diversity of bacteria associated with natural populations of *Drosophila melanogaster*. *Appl Environ Microbiol* **73**, 3470–3479 (2007).
74. Kim, G., Huang, J. H., McMullen, J. G., Newell, P. D. & Douglas, A. E. Physiological responses of insects to microbial fermentation products: Insights from the interactions between *Drosophila* and acetic acid. *J Insect Physiol* **106**, 13–19 (2018).
75. Petkau, K., Fast, D., Duggal, A. & Foley, E. Comparative evaluation of the genomes of three common *Drosophila*-associated bacteria. *Biol Open* **5**, 1305–1316 (2016).
76. Winans, N. J. *et al.* A genomic investigation of ecological differentiation between free-living and *Drosophila*-associated bacteria. *Mol Ecol* **26**, 4536–4550 (2017).

77. Martínez-Sañudo, I. *et al.* Metagenomic analysis reveals changes of the *Drosophila suzukii* microbiota in the newly colonized regions. *Insect Sci* **25**, 833–846 (2018).
78. Lin, Q. *et al.* Analyses of the gut bacteriomes of four important *Drosophila* pests. *Canadian Entomologist* **153**, 757–773 (2021).
79. Jiménez-Padilla, Y., Esan, E. O., Floate, K. D. & Sinclair, B. J. Persistence of diet effects on the microbiota of *Drosophila suzukii* (Diptera: Drosophilidae). *Canadian Entomologist* **152**, 516–531 (2020).
80. Bing, X., Gerlach, J., Loeb, G. & Buchon, N. Nutrient-Dependent Impact of Microbes on *Drosophila suzukii* Development. *mBio* **9**, e02199-17 (2018).
81. Vacchini, V. *et al.* Bacterial diversity shift determined by different diets in the gut of the spotted wing fly *Drosophila suzukii* is primarily reflected on acetic acid bacteria. *Environ Microbiol Rep* **9**, 91–103 (2017).
82. Solomon, G. M., Dodangoda, H., McCarthy-Walker, T. T., Ntim-Gyakari, R. R. & Newell, P. D. The microbiota of *Drosophila suzukii* influences the larval development of *Drosophila melanogaster*. *PeerJ* **7**, e8097 (2019).
83. Kersters, K., Lisdiyanti, P., Komagata, K. & Swings, J. The Family Acetobacteraceae: The Genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*. *The Prokaryotes* **5**, 163–200 (2006).
84. Crotti, E. *et al.* Acetic acid bacteria, newly emerging symbionts of insects. *Appl Environ Microbiol* **76**, 6963–6970 (2010).
85. Keeseey, I. W., Knaden, M. & Hansson, B. S. Olfactory Specialization in *Drosophila suzukii* Supports an Ecological Shift in Host Preference from Rotten to Fresh Fruit. *J Chem Ecol* **41**, 121–128 (2015).
86. Lauzon, C. R., McCombs, S. D., Potter, S. E. & Peabody, N. C. Establishment and Vertical Passage of *Enterobacter (Pantoea) Agglomerans* and *Klebsiella pneumoniae* through All Life Stages of the Mediterranean Fruit Fly (Diptera: Tephritidae). *Ann Entomol Soc Am* **102**, 85–95 (2009).
87. Xia, X. *et al.* Metagenomic Sequencing of Diamondback Moth Gut Microbiome Unveils Key Holobiont Adaptations for Herbivory. *Front Microbiol* **8**, 663 (2017).
88. Smith, C. C., Srygley, R. B., Healy, F., Swaminath, K. & Mueller, U. G. Spatial structure of the mormon cricket gut microbiome and its predicted contribution to nutrition and immune function. *Front Microbiol* **8**, 801 (2017).

89. Acevedo, F. E. *et al.* Fall armyworm-associated gut bacteria modulate plant defense responses. *Molecular Plant-Microbe Interactions* **30**, 127–137 (2017).
90. Raza, M. F., Yao, Z., Bai, S., Cai, Z. & Zhang, H. Tephritidae fruit fly gut microbiome diversity, function and potential for applications. *Bull Entomol Res* **110**, 423–437 (2020).
91. Singhal, K., Khanna, R. & Mohanty, S. Is *Drosophila*-microbe association species-specific or region specific? A study undertaken involving six Indian *Drosophila* species. *World J Microbiol Biotechnol* **33**, 103 (2017).
92. Martinson, V. G., Douglas, A. E. & Jaenike, J. Community structure of the gut microbiota in sympatric species of wild *Drosophila*. *Ecol Lett* **20**, 629–639 (2017).
93. Bost, A. *et al.* How gut transcriptional function of *Drosophila melanogaster* varies with the presence and composition of the gut microbiota. *Mol Ecol* **27**, 1848–1859 (2018).
94. Bourlond, B., Croxatto, A., Donner, V., Prodhom, G. & Guery, B. *Tatumella ptyseos* Causing Human Sepsis: Report of the First Case in Switzerland and Review of the Literature. *SN Compr Clin Med* **1**, 200–202 (2018).
95. Tracz, D. M. *et al.* *Tatumella saanichensis* sp. nov., isolated from a cystic fibrosis patient. *Int J Syst Evol Microbiol* **65**, 1959–1966 (2015).
96. Hollis, D. G. *et al.* *Tatumella ptyseos* gen. nov., sp. nov., a member of the family Enterobacteriaceae found in clinical specimens. *J Clin Microbiol* **14**, 79–88 (1981).
97. Adair, K. L., Wilson, M., Bost, A. & Douglas, A. E. Microbial community assembly in wild populations of the fruit fly *Drosophila melanogaster*. *ISME J* **12**, 959–972 (2018).
98. Shu, R. *et al.* Sex-Dependent Effects of the Microbiome on Foraging and Locomotion in *Drosophila suzukii*. *Front Microbiol* **12**, 656406 (2021).
99. Qiao, N. *et al.* After the storm—Perspectives on the taxonomy of Lactobacillaceae. *JDS Communications* **3**, 222–227 (2022).
100. Walter, J. Ecological role of lactobacilli in the gastrointestinal tract: Implications for fundamental and biomedical research. *Appl Environ Microbiol* **74**, 4985–4996 (2008).
101. Petri, D., Hill, J. E. & van Kessel, A. G. Microbial succession in the gastrointestinal tract (GIT) of the preweaned pig. *Livest Sci* **133**, 107–109 (2010).

102. Cholewińska, P. *et al.* Influence of selected factors on the Firmicutes, Bacteroidetes phyla and the Lactobacillaceae family in the digestive tract of sheep. *Sci Rep* **11**, 1–18 (2021).
103. McMullen, J. G., Bueno, E., Blow, F. & Douglas, A. E. Genome-Inferred Correspondence between Phylogeny and Metabolic Traits in the Wild *Drosophila* Gut Microbiome. *Genome Biol Evol* **13**, evab127 (2021).
104. Storelli, G. *et al.* *Lactobacillus plantarum* promotes *Drosophila* systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. *Cell Metab* **14**, 403–414 (2011).
105. Erkosar, B. *et al.* Pathogen Virulence Impedes Mutualist-Mediated Enhancement of Host Juvenile Growth via Inhibition of Protein Digestion. *Cell Host Microbe* **18**, 445 (2015).
106. Jones, R. M. *et al.* Symbiotic lactobacilli stimulate gut epithelial proliferation via Nox-mediated generation of reactive oxygen species. *EMBO J* **32**, 3017 (2013).
107. Jones, R. M. *et al.* Symbiotic lactobacilli stimulate gut epithelial proliferation via Nox-mediated generation of reactive oxygen species. *EMBO Journal* **32**, 3017–3028 (2013).
108. Jones, R. M. *et al.* Lactobacilli Modulate Epithelial Cytoprotection through the Nrf2 Pathway. *Cell Rep* **12**, 1217–1225 (2015).
109. Fast, D., Duggal, A. & Foley, E. Monoassociation with *Lactobacillus plantarum* disrupts intestinal homeostasis in adult *Drosophila melanogaster*. *mBio* **9**, e01114-18 (2018).
110. Trinder, M. *et al.* Probiotic *Lactobacillus rhamnosus* Reduces Organophosphate Pesticide Absorption and Toxicity to *Drosophila melanogaster*. *Appl Environ Microbiol* **82**, 6204 (2016).
111. Hiebert, N. *et al.* The Gram-Positive Bacterium *Leuconostoc pseudomesenteroides* Shows Insecticidal Activity against Drosophilid and Aphid Pests. *Insects* **11**, 1–15 (2020).
112. Sommer, A. J. & Newell, P. D. Metabolic basis for mutualism between gut bacteria and its impact on the *Drosophila melanogaster* host. *Appl Environ Microbiol* **85**, e01882-18 (2019).
113. Newell, P. D. & Douglas, A. E. Interspecies Interactions Determine the Impact of the Gut Microbiota on Nutrient Allocation in *Drosophila melanogaster*. *Appl Environ Microbiol* **80**, 788–796 (2014).

114. Rudman, S. M. *et al.* Microbiome composition shapes rapid genomic adaptation of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **116**, 20025–20032 (2019).
115. Gould, A. L. *et al.* Microbiome interactions shape host fitness. *Proc Natl Acad Sci U S A* **115**, E11951–E11960 (2018).
116. Mazzi, D., Bravin, E., Meraner, M., Finger, R. & Kuske, S. Economic Impact of the Introduction and Establishment of *Drosophila suzukii* on Sweet Cherry Production in Switzerland. *Insects* **2017** **8**, 18 (2017).
117. Bolda, M. P., Goodhue, R. E. & Zalom, F. G. Spotted Wing *Drosophila*: Potential Economic Impact of a Newly Established Pest. *ARE Update* **13**, 5–8 (2010).
118. Schöneberg, T. *et al.* Cultural Control of *Drosophila suzukii* in Small Fruit—Current and Pending Tactics in the U.S. *Insects* **12**, 172 (2021).
119. Schetelig, M. F. *et al.* Environmentally sustainable pest control options for *Drosophila suzukii*. *Journal of Applied Entomology* **142**, 3–17 (2018).
120. Clymans, R. *et al.* Mass Trapping *Drosophila suzukii*, What Would It Take? A Two-Year Field Study on Trap Interference. *Insects* **13**, 240 (2022).
121. Spies, J. M. & Liburd, O. E. Comparison of Attractants, Insecticides, and Mass Trapping for Managing *Drosophila suzukii* (Diptera: Drosophilidae) in Blueberries. *Florida Entomologist* **102**, 315–321 (2019).
122. Sassù, F. *et al.* Mass-Rearing of *Drosophila suzukii* for Sterile Insect Technique Application: Evaluation of Two Oviposition Systems. *Insects* **10**, 448 (2019).
123. Lanouette, G. *et al.* The sterile insect technique for the management of the spotted wing *Drosophila*, *Drosophila suzukii*: Establishing the optimum irradiation dose. *PLoS One* **12**, e0180821 (2017).
124. Wang, X. Biological control of *Drosophila suzukii*. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* **15**, (2020).
125. Dam, D., Molitor, D. & Beyer, M. Natural compounds for controlling *Drosophila suzukii*. A review. *Agron Sustain Dev* **39**, 1–17 (2019).
126. Cahenzli, F., Strack, T. & Daniel, C. Screening of 25 different natural crop protection products against *Drosophila suzukii*. *Journal of Applied Entomology* **142**, 563–577 (2018).

127. de Souza, M. T. *et al.* Chemical composition of essential oils of selected species of Piper and their insecticidal activity against *Drosophila suzukii* and *Trichopria anastrephae*. *Environmental Science and Pollution Research* **27**, 13056–13065 (2020).
128. Ozoe, Y. γ -Aminobutyrate- and Glutamate-gated Chloride Channels as Targets of Insecticides. *Adv In Insect Phys* **44**, 211–286 (2013).
129. Nauen, R., Slater, R., Sparks, T. C., Elbert, A. & Mccaffery, A. IRAC: Insecticide Resistance and Mode-of-action Classification of Insecticides. *Modern Crop Protection Compounds* **3**, 995–1012 (2019).
130. Suiter, D. R. & Scharf, M. E. *Insecticide Basics for the Pest Management Professional*. Univeristy of Gerogia (2009).
131. Farm to Fork Strategy. https://food.ec.europa.eu/horizontal-topics/farm-fork-strategy_en.
132. Sánchez-Bayo, F. Insecticides Mode of Action in Relation to Their Toxicity to Non-Target Organisms. *J Environ Anal Toxicol* **4**, S4-002 (2012).
133. Schlesener, D. C. H. *et al.* Insecticide Toxicity to *Drosophila suzukii* (Diptera: Drosophilidae) parasitoids: *Trichopria anastrephae* (Hymenoptera: Diapriidae) and *Pachycrepoideus vindemmiae* (Hymenoptera: Pteromalidae). *J Econ Entomol* **112**, 1197–1206 (2019).
134. Ricupero, M., Desneux, N., Zappalà, L. & Biondi, A. Target and non-target impact of systemic insecticides on a polyphagous aphid pest and its parasitoid. *Chemosphere* **247**, 125728 (2020).
135. Iwasaki, J. M. & Hogendoorn, K. Non-insecticide pesticide impacts on bees: A review of methods and reported outcomes. *Agric Ecosyst Environ* **314**, 107423 (2021).
136. Lidova, J., Buric, M., Kouba, A. & Velisek, J. Acute toxicity of two pyrethroid insecticides for five non-indigenous crayfish species in Europe. *Vet Med (Praha)* **64**, 125–133 (2019).
137. Beketov, M. A. Comparative Sensitivity to the Insecticides Deltamethrin and Esfenvalerate of Some Aquatic Insect Larvae (Ephemeroptera and Odonata) and *Daphnia magna*. *Russ J Ecol* **35**, 200–204 (2004).
138. Little, C. M., Little, C. M., Chapman, T. W. & Hillier, N. K. Plasticity Is Key to Success of *Drosophila suzukii* (Diptera: Drosophilidae) Invasion. *Journal of Insect Science* **20**, 5 (2020).

139. Bruck, D. J. *et al.* Laboratory and field comparisons of insecticides to reduce infestation of *Drosophila suzukii* in berry crops. *Pest Manag Sci* **67**, 1375–1385 (2011).
140. Disi, J. O. & Sial, A. A. Laboratory Selection and Assessment of Resistance Risk in *Drosophila suzukii* (Diptera: Drosophilidae) to Spinosad and Malathion. *Insects* **12**, 794 (2021).
141. Smirle, M. J., Zurowski, C. L., Ayyanath, M. M., Scott, I. M. & MacKenzie, K. E. Laboratory studies of insecticide efficacy and resistance in *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) populations from British Columbia, Canada. *Pest Manag Sci* **73**, 130–137 (2017).
142. Shower, R., Tonina, L., Tirello, P., Duso, C. & Mori, N. Laboratory and field trials to identify effective chemical control strategies for integrated management of *Drosophila suzukii* in European cherry orchards. *Crop Protection* **103**, 73–80 (2018).
143. van Timmeren, S., Mota-Sanchez, D., Wise, J. C. & Isaacs, R. Baseline susceptibility of spotted wing *Drosophila* (*Drosophila suzukii*) to four key insecticide classes. *Pest Manag Sci* **74**, 78–87 (2018).
144. Sial, A. A. *et al.* Evaluation of organic insecticides for management of spotted-wing *Drosophila* (*Drosophila suzukii*) in berry crops. *Journal of Applied Entomology* **143**, 593–608 (2019).
145. van Timmeren, S., Sial, A. A., Lanka, S. K., Spaulding, N. R. & Isaacs, R. Development of a rapid assessment method for detecting insecticide resistance in spotted wing *Drosophila* (*Drosophila suzukii* Matsumura). *Pest Manag Sci* **75**, 1782–1793 (2019).
146. Girod, P. *et al.* Host specificity of Asian parasitoids for potential classical biological control of *Drosophila suzukii*. *J Pest Sci (2004)* **91**, 1241–1250 (2018).
147. Sario, S., Santos, C., Gonçalves, F. & Torres, L. DNA screening of *Drosophila suzukii* predators in berry field orchards shows new predatory taxonomical groups. *PLoS One* **16**, e0249673 (2021).
148. Vega, F. E. *et al.* Fungal entomopathogens: new insights on their ecology. *Fungal Ecol* **2**, 149–159 (2009).
149. dos Santos, J. J. *et al.* Effectiveness of *Steinernema rarum* PAM 25 (Rhabditida: Steinernematidae) Against *Drosophila suzukii* (Diptera: Drosophilidae). *J Econ Entomol* **115**, 967–971 (2022).

150. Woltz, J. M., Donahue, K. M., Bruck, D. J. & Lee, J. C. Efficacy of commercially available predators, nematodes and fungal entomopathogens for augmentative control of *Drosophila suzukii*. *Journal of Applied Entomology* **139**, 759–770 (2015).
151. Lee, J. C. et al. Biological Control of Spotted-Wing *Drosophila* (Diptera: Drosophilidae)—Current and Pending Tactics. *J Integr Pest Manag* **10**, 13 (2019).
152. Malaikozhundan, B. & Vinodhini, J. Biological control of the Pulse beetle, *Callosobruchus maculatus* in stored grains using the entomopathogenic bacteria, *Bacillus thuringiensis*. *Microb Pathog* **114**, 139–146 (2018).
153. Valtierra-De-luis, D., Villanueva, M., Berry, C. & Caballero, P. Potential for *Bacillus thuringiensis* and Other Bacterial Toxins as Biological Control Agents to Combat Dipteran Pests of Medical and Agronomic Importance. *Toxins (Basel)* **12**, 773 (2020).
154. Jakka, S. R. K., Shrestha, R. B. & Gassmann, A. J. Broad-spectrum resistance to *Bacillus thuringiensis* toxins by western corn rootworm (*Diabrotica virgifera virgifera*). *Sci Rep* **6**, 1–9 (2016).
155. Bing, X. L., Winkler, J., Gerlach, J., Loeb, G. & Buchon, N. Identification of natural pathogens from wild *Drosophila suzukii*. *Pest Manag Sci* **77**, 1594–1606 (2021).
156. de Bortoli, C. P. & Jurat-Fuentes, J. L. Mechanisms of resistance to commercially relevant entomopathogenic bacteria. *Curr Opin Insect Sci* **33**, 56–62 (2019).
157. Satta, A. & Floris, I. Emerging entomopathogenic bacteria for insect pest management. *Bull Insectology* **66**, 181–186 (2013).
158. Ruiju, L. Insect Pathogenic Bacteria in Integrated Pest Management. *Insects* **6**, 352–367 (2015).
159. Khanna, A., Khanna, M. & Aggarwal, A. *Serratia Marcescens*- A Rare Opportunistic Nosocomial Pathogen and Measures to Limit its Spread in Hospitalized Patients. *J Clin Diagn Res* **7**, 243 (2013).
160. Tena, D., Fernández, C. & Lago, M. R. *Alcaligenes faecalis*: an unusual cause of skin and soft tissue infection. *Jpn J Infect Dis* **68**, 128–130 (2015).
161. Marion-Sanchez, K., Pailla, K., Olive, C., le Coutour, X. & Derancourt, C. *Achromobacter* spp. healthcare associated infections in the French West Indies: A longitudinal study from 2006 to 2016. *BMC Infect Dis* **19**, 1–10 (2019).

162. Li, G. *et al.* Complete genome sequence of *Achromobacter spanius* type strain DSM 23806 T, a pathogen isolated from human blood. *J Glob Antimicrob Resist* **14**, 1–3 (2018).
163. de Gregorio, E., Spellman, P. T., Tzou, P., Rubin, G. M. & Lemaitre, B. The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *EMBO J* **21**, 2568–2579 (2002).
164. Marra, A., Hanson, M. A., Kondo, S., Erkosar, B. & Lemaitre, B. *Drosophila* Antimicrobial Peptides and Lysozymes Regulate Gut Microbiota Composition and Abundance. *mBio* **12**, (2021).
165. Castillo, J. C. *et al.* *Drosophila* anti-nematode and antibacterial immune regulators revealed by RNA-Seq. *BMC Genomics* **16**, 1–21 (2015).
166. Capo, F., Wilson, A. & di Cara, F. The Intestine of *Drosophila melanogaster*: An Emerging Versatile Model System to Study Intestinal Epithelial Homeostasis and Host-Microbial Interactions in Humans. *Microorganisms* **7**, 336 (2019).
167. Buchon, N., Silverman, N. & Cherry, S. Immunity in *Drosophila melanogaster* — from microbial recognition to whole-organism physiology. *Nat Rev Immunol* **14**, 796–810 (2014).
168. Hoffmann, J. A. & Reichhart, J. M. *Drosophila* innate immunity: an evolutionary perspective. *Nat Immunol* **3**, 121–126 (2002).
169. Vesala, L., Hultmark, D. & Valanne, S. Editorial: Recent Advances in *Drosophila* Cellular and Humoral Innate Immunity. *Front Immunol* **11**, 598618 (2020).
170. Paquette, N. *et al.* Caspase Mediated Cleavage, IAP Binding and Ubiquitination: Linking Three Mechanisms Crucial for *Drosophila* NF-κB Signaling. *Mol Cell* **37**, 172 (2010).
171. Lemaitre, B., Reichhart, J. M. & Hoffmann, J. A. *Drosophila* host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc Natl Acad Sci U S A* **94**, 14614 (1997).
172. Myllymäki, H., Valanne, S. & Rämet, M. The *Drosophila* Imd Signaling Pathway. *The Journal of Immunology* **192**, 3455–3462 (2014).
173. Choe, K. M., Werner, T., Stöven, S., Hultmark, D. & Anderson, K. v. Requirement for a peptidoglycan recognition protein (PGRP) in relish activation and antibacterial immune responses in *Drosophila*. *Science* (1979) **296**, 359–362 (2002).

174. Takehana, A. *et al.* Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in *Drosophila* larvae. *Proc Natl Acad Sci U S A* **99**, 13705–13710 (2002).
175. Hultmark, D. *Drosophila* immunity: paths and patterns. *Curr Opin Immunol* **15**, 12–19 (2003).
176. Bischoff, V. *et al.* Downregulation of the *Drosophila* Immune Response by Peptidoglycan-Recognition Proteins SC1 and SC2. *PLoS Pathog* **2**, e14 (2006).
177. Combe, B. E. *et al.* *Drosophila* Microbiota Modulates Host Metabolic Gene Expression via IMD/NF- κ B Signaling. *PLoS One* **9**, 94729 (2014).
178. Kleino, A. *et al.* Pirk is a negative regulator of the *Drosophila* Imd pathway. *J Immunol* **180**, 5413–5422 (2008).
179. Kim, S. H. & Lee, W. J. Role of DUOX in gut inflammation: Lessons from *Drosophila* model of gut-microbiota interactions. *Front Cell Infect Microbiol* **3**, 116 (2014).
180. Ha, E. M. *et al.* Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in *Drosophila* gut. *Nat Immunol* **10**, 949–957 (2009).
181. Lee, K. A. *et al.* Bacterial-derived uracil as a modulator of mucosal immunity and gut-microbe homeostasis in *Drosophila*. *Cell* **153**, 797–811 (2013).
182. Ha, E. M. *et al.* Regulation of DUOX by the G α q-Phospholipase C β -Ca²⁺ Pathway in *Drosophila* Gut Immunity. *Dev Cell* **16**, 386–397 (2009).
183. Brun, S. *et al.* The MAPKKK Mekk1 regulates the expression of Turandot stress genes in response to septic injury in *Drosophila*. *Genes to Cells* **11**, 397–407 (2006).
184. Valanne, S., Wang, J.-H. & R  met, M. The *Drosophila* Toll Signaling Pathway . *The Journal of Immunology* **186**, 649–656 (2011).
185. Gobert, V. *et al.* Dual Activation of the *Drosophila* Toll Pathway by Two Pattern Recognition Receptors. *Science* (1979) **302**, 2126–2130 (2003).
186. Gottar, M. *et al.* Dual Detection of Fungal Infections in *Drosophila* via Recognition of Glucans and Sensing of Virulence Factors. *Cell* **127**, 1425–1437 (2006).

187. Rushlow, C. A., Han, K., Manley, J. L. & Levine, M. The Graded Distribution of the dorsal Morphogen Is Initiated by Selective Nuclear Transport in *Drosophila*. *Cell* **59**, 1165–177 (1969).
188. Ip, Y. T. *et al.* Dif, a dorsal-related gene that mediates an immune response in *Drosophila*. *Cell* **75**, 753–763 (1993).
189. Meng, X., Khanuja, B. S. & Ip, Y. T. Toll receptor-mediated *Drosophila* immune response requires Dif, an NF- κ B factor. *Genes Dev* **13**, 792–797 (1999).
190. Carrau, T. *et al.* The Cellular Innate Immune Response of the Invasive Pest Insect *Drosophila suzukii* against *Pseudomonas entomophila* Involves the Release of Extracellular Traps. *Cells* **10**, 3320 (2021).
191. Garriga, A., Mastore, M., Morton, A., del Pino, F. G. & Brivio, M. F. Immune Response of *Drosophila suzukii* Larvae to Infection with the Nematobacterial Complex *Steinernema carpocapsae*–*Xenorhabdus nematophila*. *Insects* **11**, 210 (2020).
192. Kim, Y., Goh, G. & Kim, Y. H. Expression of antimicrobial peptides associated with different susceptibilities to environmental chemicals in *Drosophila suzukii* and *Drosophila melanogaster*. *Pestic Biochem Physiol* **187**, 105210 (2022).
193. Deng, D. *et al.* Transcriptome analysis of sex-biased gene expression in the spotted-wing *Drosophila*, *Drosophila suzukii* (Matsumura). *G3 Genes|Genomes|Genetics* **12**, (2022).
194. Marasco, R. *et al.* Destabilization of the Bacterial Interactome Identifies Nutrient Restriction-Induced Dysbiosis in Insect Guts. *Microbiol Spectr* **10**, e01580-21 (2022).
195. Daisley, B. A., Chmiel, J. A., Pitek, A. P., Thompson, G. J. & Reid, G. Missing Microbes in Bees: How Systematic Depletion of Key Symbionts Erodes Immunity. *Trends Microbiol* **28**, 1010–1021 (2020).
196. Wu, Y. *et al.* Honey bee (*Apis mellifera*) gut microbiota promotes host endogenous detoxification capability via regulation of P450 gene expression in the digestive tract. *Microb Biotechnol* **13**, 1201–1212 (2020).
197. Buchon, N., Broderick, N. A., Chakrabarti, S. & Lemaitre, B. Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*. *Genes Dev* **23**, 2333–2344 (2009).
198. Shin, S. C. *et al.* *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science* (1979) **334**, 670–674 (2011).

199. Henry, Y. & Colinet, H. Microbiota disruption leads to reduced cold tolerance in *Drosophila* flies. *Science of Nature* **105**, 1–5 (2018).
200. Steinigeweg, C. *et al.* Impact of a Microbial Pest Control Product Containing *Bacillus thuringiensis* on Brood Development and Gut Microbiota of *Apis mellifera* Worker Honey Bees. *Microb Ecol* **1**, 1–8 (2022).
201. Marasco, R. *et al.* Destabilization of the Bacterial Interactome Identifies Nutrient Restriction-Induced Dysbiosis in Insect Guts. *Microbiol Spectr* **10**, e01580-21 (2022).
202. Ludington, W. B. & Ja, W. W. *Drosophila* as a model for the gut microbiome. *PLoS Pathog* **16**, e1008398 (2020).
203. Wong, A. C. N., Dobson, A. J. & Douglas, A. E. Gut microbiota dictates the metabolic response of *Drosophila* to diet. *Journal of Experimental Biology* **217**, 1894–1901 (2014).
204. Ankrah, N. Y. D. *et al.* Predicted Metabolic Function of the Gut Microbiota of *Drosophila melanogaster*. *mSystems* **6**, e01369-20 (2021).
205. Pais, I. S., Valente, R. S., Sporniak, M. & Teixeira, L. *Drosophila melanogaster* establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. *PLoS Biol* **16**, e2005710 (2018).
206. Fountain, M. T. *et al.* Alimentary microbes of winter-form *Drosophila suzukii*. *Insect Mol Biol* **27**, 383–392 (2018).

I.II. Hypothesis & Objectives

The *Drosophila suzukii* pest is a current reality which threatens producers and the agroindustry. Novel and more sustainable strategies are demanded for the efficient control and mitigation of damages caused by this pest. Although for the past decade efforts have been concentrated on the characterization of this species and development of biocontrol tools, there is still some gaps which need to be fulfilled to achieve the main goal.

Therefore, this dissertation is in line with three main objectives, (**Chapter II**) to characterize the microbiome of Portuguese populations by employing metagenomic and isolation of microorganisms; (**Chapter III**) to test new putative natural entomopathogenic bacteria towards *D. suzukii* and evaluate both the insecticidal activity, as well as the host's immune molecular response to characterize relevant mechanisms of resistance; and (**Chapter IV**) to develop a method for the study of colonizing ability of bacteria in the gut of *D. suzukii* to understand the structure of the microbial community of this species.

We hypothesize that by harnessing the potential of bacteria and the microbiome as an important physiological structure, new strategies may be developed to respond to a worldwide problem in a more sustainable and biological way.

II. Chapter 2: Unveiling Portuguese *Drosophila suzukii* microbiome assembly: do sex and food supply matter?

Abstract

The insect gut microbiome is important as a model in insect models or as a target for developing targeted biocontrol tools against pests, like *Drosophila suzukii*, and the study of this pest microbiomes has recently emerged. This study aimed to characterize the gut microbiome assembly of *D. suzukii* populations sampled in Portugal in 2021, using both cultivable tools (isolation and phenotypic characterization), and metagenomic tools (for characterizing the gut microbiome genetic structure regarding key taxa). We also aimed to clarify the most abundant taxa and diversity present in wild *D. suzukii* adults and larva, adults emerged from field fruits (raspberry and blueberry) in 2021 and laboratory-reared adults. The ultimate goal was to fill the gap in literature regarding the establishment of a core microbiome of this species by comparing all samples. 16S rRNA metagenomic data showed Proteobacteria as the most prominent phylum in all samples, and that laboratory-reared flies, females fed on raspberry, and males fed on blueberry, distanced from remaining samples, possibly suggesting a synergetic interaction between sex and diet factors. Moreover, Acetobacteraceae (acetic acid bacteria) family occurred in all samples, suggesting a strong association with *D. suzukii*, and integrating its core microbiome. Isolation of microorganisms also revealed a high prominence of *Frigoribacterium*, a genus also common in soil-samples, suggesting a possible new genus associated with this pest. Also, the isolation of *Tatumella* supports the association of this genus with *D. suzukii* larvae. This approach filled some gaps and further consolidated information about the *D. suzukii* microbiome for future targeted manipulation and use in biocontrol strategies.

1. Introduction

The concept of holobiont emerged from the recognition that several host-microorganism links confer mutualistic advantages and a parallel evolution to animals, from vertebrates to invertebrates^{1,2}. Although contact with some microorganisms may lead to diseases in the host, most microorganisms are innocuous or indispensable for the host's physiological regulation, metabolic processes, neuronal development, and homeostasis³⁻⁵. Although the microbiome occurs in multiple parts and organs of the

host, the gut microbiota has been the most widely studied in humans and other animals⁶.

Invertebrates, namely insects, also associate with microbes and, a gut-microbiome has already been identified in several species namely *Drosophila melanogaster* and more recently in *D. suzukii*^{7,8}. The identification of gut-associated microbial genetic structure usually shows a core of specialized bacteria being encountered in all individuals of the same species, while the presence and abundance of other taxa often differ between individuals⁹, apparently dependent on the individual's sex, age, food supply, environmental conditions, etc^{10,11}. Thus, researchers currently believe that a "core" consortium of microbiome taxa may be characteristic of a species¹². Besides this core consortium, the microbiome contains other taxa that establish more transient associations with each other and the host^{13,14}. Detoxification, protection, development, and nutritional advantages are some properties that some gut microbial populations may provide to insect hosts^{9,15–17}. Also, microbial populations form positive, neutral, or negative interactions with other populations which maintain the microbiome homeostasis in nature. Shifts to negative interactions (due to e.g., pathogens) may lead to dysbiosis¹⁸. Thus, due to its significant importance in homeostasis maintenance, insect gut microbiota gained momentum among researchers as a potential target for biotechnological application and the development of biocontrol tools. For example, finding microbial species acting as antagonists of pathogens, or targeting specific symbionts or paratransgenesis^{19–24} emerge as promising techniques to control pathogens or pests.

The development of new sequencing platforms, such as next-generation sequencing (NGS), attained a high-throughput characterization of several insects' associated microbes. Extensive 16S ribosomal RNA (rRNA) DNA metabarcoding has been employed for the study of bacterial communities in several insect pests^{25–27}. Nevertheless, the implementation of NGS along with the isolation of lab-culturable bacteria provides a synergetic approach by combining the identification of the genetic structure of the microbiome with, simultaneously, the isolation and culture of bacteria present in the microbiomes, and thus create collection of bacteria, and study for keystone bacteria with potential for new biocontrol tools¹⁹.

Drosophila suzukii is a highly polyphagous pest that currently threatens a variety of red fruits and berries²⁸. This East Asian invasive pest has settled in different countries across the European and American continents^{29–34} and predictive models reveal a wider distribution in the future³⁵. The economic impact of this pest is a challenging

issue for producers^{36–44}, who still rely mainly upon the application of pesticides to mitigate crop loss^{45–47}. Not only is the employment of pesticides a risk to public health^{48,49}, but also to environment, due to the broad spectrum of these pesticides, along with the development of resistance, lowering their efficacy^{50–52}. Therefore, the demand for new control strategies has been increasing. Biological control of *D. suzukii* has been in the scope of scientific studies as a potential alternative to pesticides⁵³. Particularly, harnessing the potential of entomopathogenic bacteria has been studied, namely *Bacillus thuringiensis*, a commercially available entomopathogen⁵⁴, and naturally isolated bacteria^{55–57}. Developing new microbial control strategies thus requires a first step of identifying *D. suzukii*-associated bacteria and establish collections of culturable isolates.

Previous studies dwelled on the characterization of this pest's microbiome, combining efforts to establish its core microbiota^{55,56,58–64}. Contrary to *D. melanogaster*, a closely related species and a widely used biological model for microbiome research with a thoroughly studied microbiome, *D. suzukii* still lacks a well-identified microbiota⁷, due to high variability between samples and individuals. In fact, a lack of core microbiota in the gut of *D. suzukii* has been proposed due to the high variability of the microbial communities found in different reports and between samples⁵⁸.

Chandler *et al.* (2014) carried the first characterization of *D. suzukii* bacterial communities, disclosing a dominance of *Tatumella* genus, followed by a high abundance of acetic acid bacteria (AAB) from the *Acetobacter* and *Gluconobacter* genera⁶². Ensuing studies have also focused on the identification of *D. suzukii* associated bacteria by 16S rRNA metagenomics and/or identification of isolated bacteria. The extensive isolation of bacteria by Hiebert *et al.* (2020) from wild *D. suzukii* samples has corroborated Chandler *et al.* (2014) by identifying *Tatumella* spp. as an abundant group^{56,65}. Conversely, Bing *et al.* (2018) found a dominance of the *Dickeya* genus in their wild samples and revealed the dominance of the Enterobacteriaceae family in laboratory-reared flies⁵⁸. AAB, belonging to the Acetobacteraceae family, have also been extensively identified in association with *D. suzukii*^{60,64} revealing a similarity with *D. melanogaster* and other *Drosophila* spp. that also associate with AAB^{7,61,66}. Moreover, *D. suzukii* undergoes a morphological shift during overwintering; Fountain *et al.* (2018) assessed the microbial composition in gut-associated microbes between summer and winter morphotypes, revealing a dominance of *Pseudomonas* spp. in winter morphs⁶³.

In order to tackle the knowledge gap on the microbiome of *D. suzukii*, this study aimed to characterize the bacterial community associated with sampled male and female flies from Portuguese orchards by employing NGS metagenomic identification and simultaneous listing of isolated bacteria. Moreover, we hypothesised that the microbiome genetic structure varied with sex and environmental conditions, namely food supply. To test this hypothesis, we characterized the bacterial communities of wild-caught *D. suzukii* adults and larva, adults that emerged from raspberry and blueberry of infested orchards and laboratory-reared flies.

2. Materials and Methods

2.1. *Drosophila suzukii* Collections

Adult wild flies from Castelo de Paiva (CP21), Portugal (41°03'14.3"N 8°16'49.2"W) were collected by aspiration using a D-VAC with an insect net in 2021. To obtain the first generation of flies in artificial fly medium (10% sucrose, 8% cornmeal, 4% yeast, 0.08% methyl 4-hydroxybenzoate, 0.15% orthophosphoric acid and 1.5% agar in distilled water), infested fruits from the previous location were placed into plastic bags filled with a sucrose solution (at an arbitrary concentration) and smashed until larvae were visible in the liquid⁶⁷. Then, larvae were filtered and placed into artificial fly medium and one larva was directly taken to bacterial isolation. To obtain flies emerging from fruits, infested raspberries and blueberries were collected from Santa Maria da Feira (SMF21), Portugal (40°55'19.5"N 8°33'09.1"W), Oliveira de Azeméis (OAZ21), Portugal (40°50'19.1"N 8°28'01.1"W) and Vila Nova de Gaia (G21), Portugal (41°01'44.5"N 8°30'23.1"W), respectively in 2021, and placed into sealed transparent containers at room temperature until the adults emerged. Adult *D. suzukii* were morphologically selected, separated by sex (females, F and males, M) and stored at -20°C. Samples collected are summarized in Supplementary Materials Table SII-6.

2.2. Bacterial Isolation and Identification

To isolate bacteria from the abdomen of sampled flies, one female and one male fly from each location were surface sterilized with 10% bleach for 10 min and 70% EtOH for 5 min and washed with sterile water. Abdomens were dissected and mashed in sterile phosphate-buffered saline (PBS) pH 7.3 and plated on Lysogeny Broth (LB) medium (Liofilchem®, Roseto degli Abruzzi, Italy) (1.5% agar), Brain Heart Infusion (BHI) (Merck, Darmstadt, Germany) (1.5% agar) and De Man, Rogosa and Sharpe (MRS) (Panreac AppliChem, Barcelona, Spain) (1.5% agar) and incubated at 25°C for up to 4 days. A colony from each different colony morphotype was isolated.

2.3. Identification of Bacterial Isolates

To obtain genomic DNA from each isolate, bacteria from a single colony were grown in LB overnight at 25°C at 180 rpm in an orbital agitator and incubator. The suspension was centrifuged for 3 min at 5000 *g* and the bacteria were then resuspended in lysis solution provided by GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit (EURx, Gdansk, Poland), and DNA purification was carried out according to the manual's instructions. 16S rRNA was amplified through PCR using the universal primers 27F (5' - GAGAGTTTGATCCTGGCTCAG - 3') and 1492R (5' - AGAGTTTGATCCTGGCTCAG - 3')⁶⁸ and the following thermal cycler program (95°C for 5 minutes; 24 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 2 minutes; 72°C for 10 minutes). The 1500 bp products were then purified from an agarose gel (1%) according to the instructions of the NZYGelpure kit (NZYTech, Lisbon, Portugal) and sent to Sanger Sequencing at STABVida, Almada, Portugal. Each isolate was identified using each retrieved sequence in the BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.4. Genomic DNA Collection for Metagenomics

Genomic DNA from *D. suzukii*-associated bacteria was obtained from a pool of 3 flies per sex and location (Castelo de Paiva, Oliveira de Azeméis, Vila Nova de Gaia, and Laboratory). Flies were first surface sterilized as previously described in section 2.2. and placed into the lysis solution of the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany), and purification was carried out following the manufacturer's instructions with 4 hours of lysis and no carrier RNA. DNA was eluted in MilliQ water. DNA concentration and absorbance ratios were then obtained using a microdrop (BMG, Berlin, Germany) and integrity was assured by assessing the samples in an agarose gel (1%).

2.5. NGS 16S rRNA Metagenomics

Amplicon 16S rRNA metagenomic analysis was outsourced to Novogene, Cambridge, United Kingdom. Libraries were generated after PCR amplification of the V3-V4 hypervariable regions of 16S rRNA gene and ligated with the proper Illumina adapters. After purification of the library, its quality was assessed using Qubit, RT-qPCR, and bioanalyzer. Samples were then sequenced using sequencing by synthesis technology in the Illumina NovaSeq 6000 platform.

2.6. Sequencing Data Analysis

Sample identifier barcodes were removed from the paired-end reads and merged into raw tags using the FLASH (V1.2.7) software⁶⁹. Cleaned tags were then generated by

the Qiime (V1.7.0) software⁷⁰ and then compared to the SILVA138 database (<http://www.arb-silva.de/>)⁷¹ with the UCHIME software⁷² to detect and remove chimera sequences, and generate the effective tags. Operational Taxonomic Units (OTUs) were assigned to effective tags with $\geq 97\%$ identity with the UPARSE (V7.0.1090) software⁷³. For each OTU, a representative sequence was retrieved for taxonomic annotation against the SSUrRNA database of the SILVA138 database (<http://www.arb-silva.de/>)⁷¹. Finally, the abundance of OTUs was normalized to the sample with the least number of sequences.

2.6. Microbial Community Structure and Alpha and Beta Diversity Analysis

Abundance graphs were generated in GraphPad Prism V8.0.0 (GraphPad Software, San Diego, California, USA) representing the top 10 most abundant genera in each sample. Rarefaction curves were generated by subsampling sequences from each sample and counting the number of OTUs, representing the richness of the community. Alpha-diversity indices (Shannon, Simpson, Chao1, and ACE) were generated using Qiime (V1.7.0)⁷⁰ and R (2.15.3) software⁷⁴. Weighted and Unweighted UniFrac beta-diversity analysis was performed with the Qiime (V1.7.0) software⁷⁰. Principal Coordinate Analysis (PCoA) was carried out by constructing a distance matrix of the UniFrac values and displayed using the WGCNA⁷⁵ and ggplot2 packages⁷⁶. Venn and flower diagrams were generated by identifying the unique and common OTUs present in the analysed samples.

3. Results

3.1. Sequencing Data and Quality

Sequencing of the various samples CP21 (Castelo de Paiva 2021, wild), G21 (Vila Nova de Gaia, blueberry fruit-emerged), OAZ21 (Oliveira de Azeméis, raspberry fruit-emerged), and Lab (Laboratory-reared) produced, on average, 97 918 reads in a total of 587 509 reads. After merging reads, tag cleaning and filtering, the number of effective tags was, on average, 75 285 tags in a total of 451 708 tags. Effective tags had an average length of 414 bp. Sequencing quality was assured as, on average, 97.84% of effective tags disclosed a Quality Score equal to or above 20 (Q20). The average number of OTUs retrieved from the effective tags was 579. Data is summarized in Table II.1.

Table II-1 - Sequencing data and quality control stats. Number of raw reads, raw tags, clean tags and effective tags (after cleaning and filtering) by sample alongside with average and sum for all samples. Average length of the effective tags, percentage of tags with quality score equal to or above 20 (Q20) and percentage of effective tags according to the number of raw reads. Number of annotated tags (assigned to a taxon), unannotated tags (with no assignment), unique tags (number of tags with a frequency of 1 unique to that sample) and number of OTUs per sample. Sample names: CP21F (Castelo de Paiva, 2021, Females), G21F (Vila Nova de Gaia, 2021, Females), G21M (Vila Nova de Gaia, 2021, Males), OAZ21F (Oliveira de Azeméis, 2021, Females), OAZ21M (Oliveira de Azeméis, 2021, Males), LabM (Laboratory-reared, Males).

Sample Name	Raw Reads	Raw Tags	Clean Tags	Effective Tags	Average Length	Q20	Effective Tags (%)	Annotated Tags	Unannotated Tags	Unique Tags	OTUs Number
CP21F	117607	105379	103987	94095	405	98.22	80.01	92443	9	1643	578
G21F	112649	85101	79555	57331	421	97.68	50.89	51033	164	6134	926
G21M	79231	61857	57478	54664	402	98.26	68.99	50965	231	3468	289
OAZ21F	119065	97849	96353	83932	405	97.84	70.49	82403	29	1500	401
OAZ21M	148789	116469	107443	101785	424	98.53	68.41	94523	736	6526	895
LabM	10168	69006	63693	59901	427	96.5	58.91	57984	105	1812	383
Average	97918	89277	84752	75285	414	98	66	71559	212	3514	579
Sum	587509	535661	508509	451708							

3.2. Bacterial Community of Wild Female *Drosophila suzukii*

Metagenomic analysis of the bacterial community in female wild-collected *D. suzukii* (Figure II.2) revealed a major dominance of the genus *Komagataeibacter* (62.2%), followed by *Acetobacter* (21.5%), which was also highly represented in this sample. Other genera, present in lower abundance, were part of the top 10: *Pseudomonas* (2.9%), *Gluconobacter* (1.9%), *Pantoea* (0.45%), *Stenotrophomonas* (0.35%), *Ralstonia* (0.17%), *Morganella* (0.14%), *Romboutsia* (0.12%) and *Bacillus* (0.12%). The remaining identified genera represented 10.2% of the community.

Despite the accentuated representativity of *Komagataeibacter* genus in the metagenomic analysis, no isolate of this genus was identified (Table II-2). However, other bacteria from the most abundant genus were isolated, such as *Acetobacter* and *Pantoea* (Table II-2). Other genera isolated bacteria from wild female *D. suzukii* include *Microbacterium*, *Rhodococcus*, *Paenibacillus*, *Leuconostoc*, *Providencia*, *Staphylococcus*, and *Burkholderia*.

Isolation of bacteria from male flies and larva (from which metagenomic analysis, was not performed), despite disclosing fewer observed species, identified other unique genera, such as *Tatumella*, *Frigoribacterium*, *Arthrobacter*, and *Pseudomonas*.

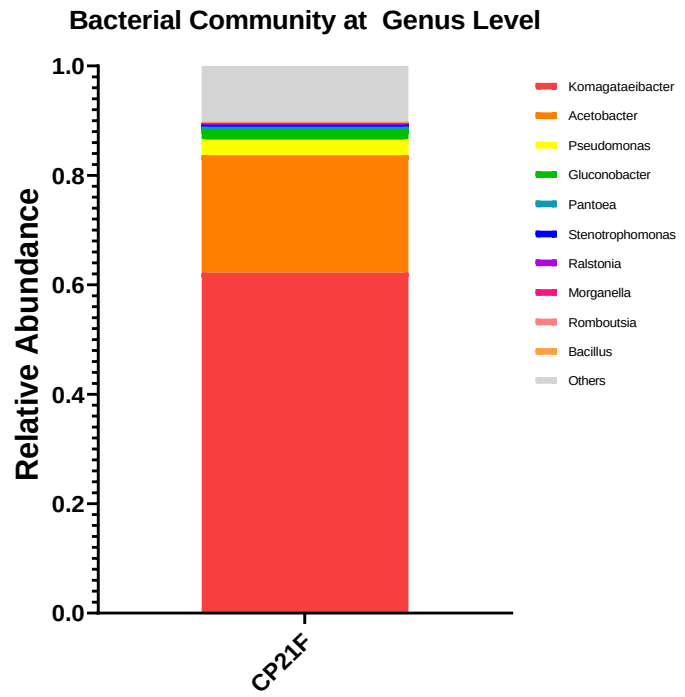


Figure II.1 - Relative abundance of genera in wild female *Drosophila suzukii*. Top 10 most representative genera in Castelo de Paiva, 2021 female (CP21F) sample.

Table II-2 – Isolates from wild-caught *Drosophila suzukii*. Different species, genera or families identified from wild-caught *D. suzukii* from Castelo de Paiva, 2021 (CP21F) according to sex (male and female) and in larva.

CP21		
Adults		Larva
Male	Female	
<i>Tatumella ptyseos</i>	<i>Pantoea</i> sp.	<i>Tatumella</i> sp.
<i>Frigoribacterium</i> sp.	<i>Microbacterium testaceum</i>	<i>Frigoribacterium</i> sp.
<i>Arthrobacter</i> sp.	<i>Acetobacter thailandicus</i>	<i>Providencia</i> sp.
<i>Pseudomonas psychrophila</i>	<i>Rhodococcus</i> sp.	
	Microbacteriaceae (family)	
	<i>Paenibacillus provencensis</i>	
	<i>Leuconostoc pseudomesenteroides</i>	
	<i>Providencia</i> sp.	
	<i>Staphylococcus</i> sp.	
	Enterobacteriaceae (family)	
	<i>Burkholderia</i> sp.	

3.4. Bacterial Communities of fruit-emerged *Drosophila suzukii* individuals

Some samples of fruit-emerged individuals of *D. suzukii* disclosed different representative genera from the wild-collected *D. suzukii* (Figure II.2A). That was the

case of females that emerged from blueberries (G21F), in which the *Pseudomonas* genus was the most prevalent (32.4%). On the contrary, males who emerged from blueberries (G21M), disclosed dominance by the *Komagataeibacter* genus (50.8%), followed by *Acetobacter* (28.7%) and *Gluconobacter* (4.2%). Similarly, females developed in raspberries (OAZ21F) also presented dominance by these three genera, namely, *Acetobacter* (69.9%), followed by *Gluconobacter* (11.1%) and *Komagataeibacter* (5.1%). In males from raspberries (OAZ21M), although *Acetobacter* (3.6%), *Burkholderia-Caballeronia-Paraburkholderia* (BCP) group (3.6%) and *Gluconobacter* (3.0%) were the most prevalent genera, there was no significant dominance by any genera, as disclosed by the representativity of other genera (85.1%). *Wolbachia* genus was present in the top 10 genera of both samples from OAZ21 (Figure II.2A).

Venn Diagram in Figure II.2B discloses the number of shared OTUs between the group of samples of *D. suzukii* developed in raspberry (OAZ21) and blueberry (G21). 25.5% of the OTUs were shared between both conditions and roughly the same percentage of OTUs was unique to each condition (raspberry: 38.4% and blueberry: 36.1%).

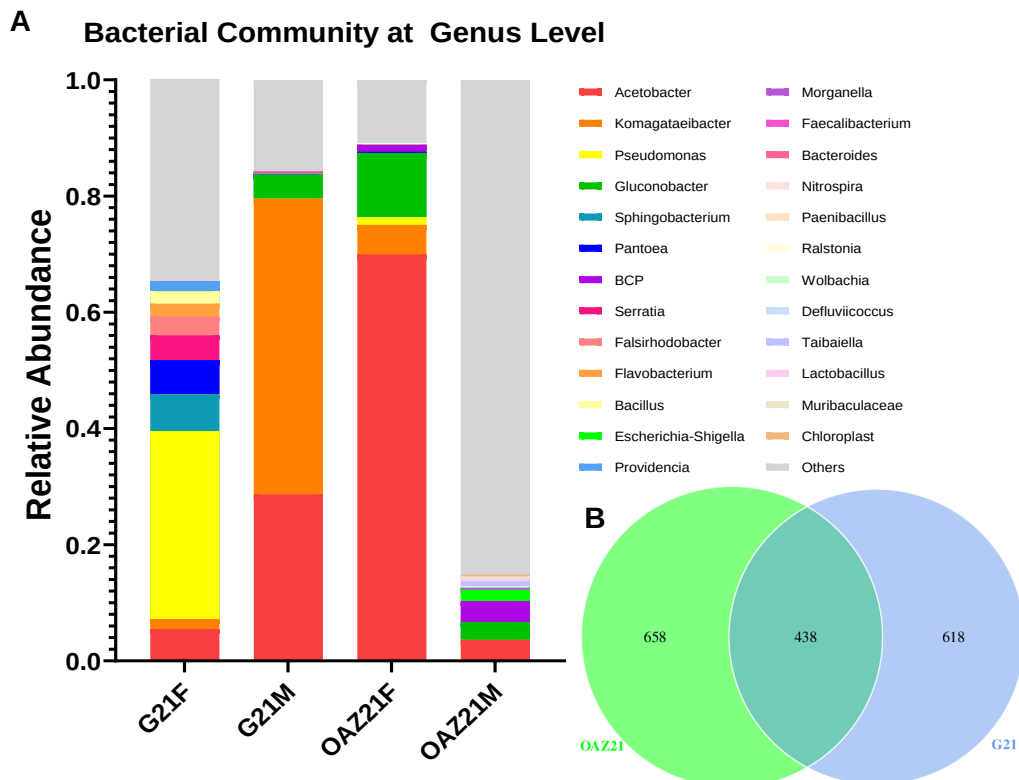


Figure II.2 - Relative abundance of genera in fruit-born *Drosophila suzukii*. (A) Top 10 most representative genera in Vila Nova de Gaia, 2021 (G21) and Oliveira de Azeméis (OAZ21) according to sex (male, M and female, F). (B) Venn diagram representing the number of shared and unique OTUs between raspberry-born (OAZ21) and blueberry-born (G21) group samples.

The bacteria isolated from individuals that emerged in raspberry (OAZ21 and SMF21) and blueberry (G21) revealed higher diversity in the samples from SMF21 (from which metagenomic analysis was not performed) (Table II-3). The isolation of bacteria confirmed the presence of the *Acetobacter*, *Pseudomonas* and *Pantoea* genera in raspberry-emerged *D. suzukii*, although from a different orchard. Other isolates from raspberry-emerged individuals belong to the genera *Frigoribacterium*, *Bacillus*, *Staphylococcus*, *Brucella*, *Cellulosimicrobium*, *Providencia* and *Microbacterium*. *Frigoribacterium*, *Alkalihalobacillus*, and *Paenibacillus* were the only genera isolated from individuals emerged from blueberries.

Table II-3 - Isolates from fruit-emerged adults of *Drosophila suzukii*. Different species, genera or families identified from fruit-born *D. suzukii* collected from Oliveira de Azeméis, 2021 (OAZ21), Santa Maria da Feira, 2021 (SMF21) and Vila Nova de Gaia, 2021 (G21), Portugal 2021 according to sex (male and female).

Raspberry		Blueberry
OAZ21	SMF21	G21
Male		
<i>Frigoribacterium</i> sp.	<i>Frigoribacterium</i> sp.	<i>Frigoribacterium</i> sp.
<i>Bacillus</i> sp.	<i>Staphylococcus epidermis</i>	
	<i>Brucella pseudogrignonensis</i>	
	<i>Cellulosimicrobium</i> sp.	
	<i>Providencia</i> sp.	
	<i>Pseudomonas endophytica</i>	
Female		
<i>Frigoribacterium</i> sp.	<i>Pantoea</i> sp.	<i>Frigoribacterium</i> sp.
	<i>Microbacterium thalassium</i>	<i>Alkalihalobacillus xiaoxiensis</i>
	<i>Acetobacter persici</i>	<i>Paenibacillus</i> sp.
	Enterobacteriaceae (family)	
	<i>Brucella pseudogrignonensis</i>	

3.5. Bacterial Community of Laboratory-reared Male *Drosophila suzukii*

Laboratory male flies displayed dominance by the *Pseudomonas* genus, with 90.4% abundance. Other genera, although much less prominent, were identified: *Ralstonia* (3.81%), *Acetobacter* (1.83%), *Komagataeibacter* (0.58%), *Gluconobacter* (0.35%), *Pantoea* (0.22%), *Serratia* (0.13%), BCP group (0.12%), *Faecalibacterium* (0.1%), *Comamonas* (0.08%) (Figure II.3).

Bacteria isolated from laboratory-reared flies disclosed the presence of the *Acetobacter* genus in both female and male flies (Table II-4). *Pseudomonas* genus, although very

prominent in the metagenomic analysis, was not identified in culture. Other genera isolated from laboratory-reared flies were *Enterobacter*, *Frateruria*, and *Microbacterium*.

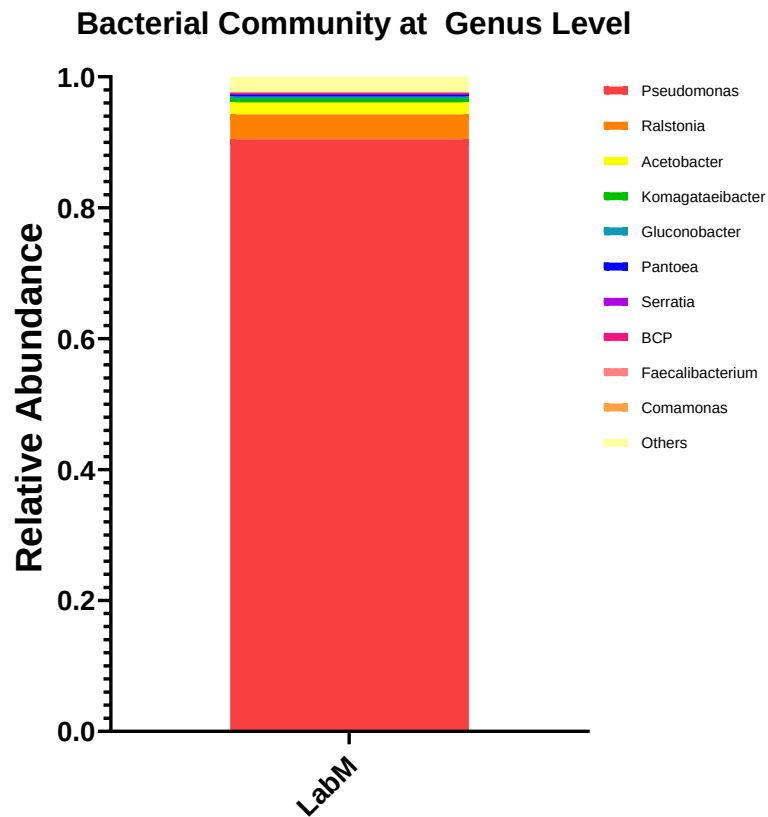


Figure II.3 - Relative abundance of genera in laboratory-reared *Drosophila suzukii*. Top 10 most representative genera in laboratory-reared male *D. suzukii* (LabM) samples.

Table II-4 - Isolates from laboratory-reared *Drosophila suzukii*. Different species or genera were identified from laboratory-reared *D. suzukii* according to sex (male and female).

Laboratory	
Male	Female
<i>Acetobacter persici</i>	<i>Microbacterium oxydans</i>
<i>Enterobacter</i> sp.	<i>Acetobacter thailandicus</i>
<i>Frateruria</i> sp.	<i>Acetobacter persici</i>

3.2. Species Richness and Alpha Diversity of Bacterial Communities

The most representative phylum was Proteobacteria (representing between 75.3% and 98.8% of the OTUs in all samples; data not shown). Rarefaction curves revealed sample G21F as harbouring the highest population diversity after a coverage of 50 000 sequences (Figure II.4). On the other hand, OAZ21M sample did not seem to reach

saturation at the obtained coverage, indicating a richer community. This observation is corroborated by the Chao1 and Abundance-based Coverage Estimator (ACE) indices, which revealed that OAZ21M harbours the highest richness in species (Chao1: 1318.461 and ACE: 1409.974), followed by G21F (Chao1: 937.258 and ACE: 941.713) and CP21F (Chao1: 654.389 and ACE: 706.965) (Table II-5). Grouped samples of raspberry-emerged individuals (OAZ21) presented higher average Chao1 (892.31) and ACE (946.19) indices than blueberry-emerged individuals (G21) (data not shown). Contrarily, grouped samples of blueberry-emerged individuals (G21) revealed higher average Shannon (4.23) and Simpson (0.838) indices than raspberry-emerged individuals (OAZ21) (data not shown).

The Shannon and Simpson indices, on the other hand, revealed that G21F bacterial community is the most diverse (Shannon: 6.135 and Simpson: 0.959), while the other samples do not distinguish from each other (Table II-5).

Moreover, it is noticeable that in both diversity and species richness, laboratory-reared flies have lower indices, revealing a less rich and diverse bacterial community, in comparison to the other samples (Figure II.4; Table II-5).

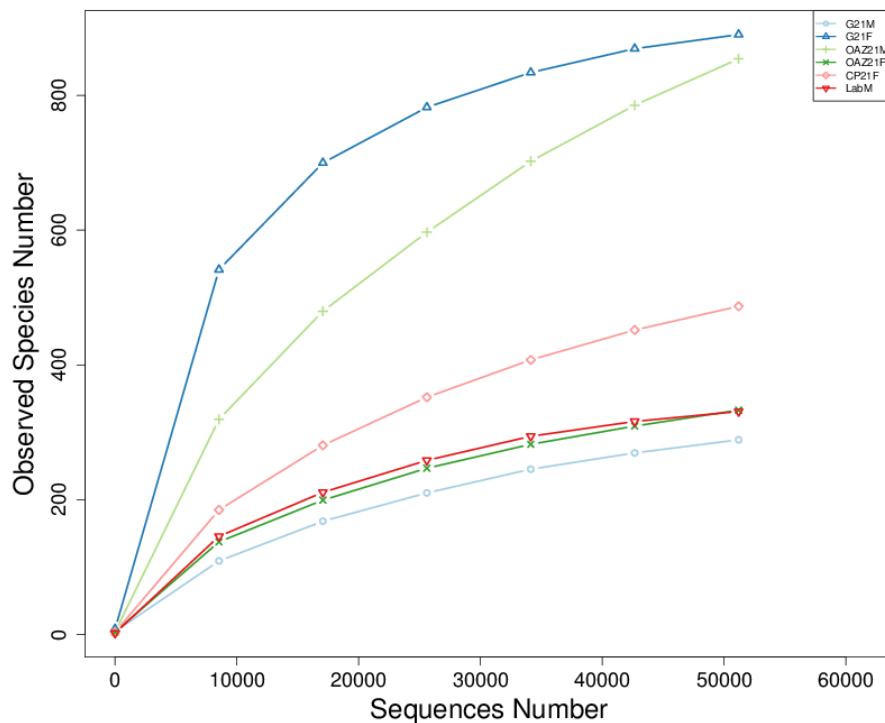


Figure II.4 - Rarefaction curves for observed species number. Number of OTUs retrieved according to the number of sequences from the different samples.

Table II-5 - Community diversity and richness indices. Number of observed species, alongside with Shannon and Simpson indices, representing diversity, and Chao1 and ACE indices representing richness of the different samples.

Sample name	Observed Species	Shannon Index	Simpson Index	Chao1	ACE
CP21F	487	2.481	0.622	654.389	706.965
G21M	289	2.63	0.717	404.412	396.407
G21F	890	6.135	0.959	937.258	941.713
OAZ21M	854	2.979	0.626	1318.461	1409.974
OAZ21F	333	2.611	0.732	466.161	482.406
LabM	331	0.971	0.199	377.974	383.833
Average	530.7	3.0	0.6	693.1	720.2

3.6. Beta-Diversity and Distance Analysis of Microbial Communities

Principal Coordinate Analysis (PCoA) of the distance matrix on weighted and unweighted UniFrac indices disclosed different outcomes about the dissimilarity between samples. While the unweighted analysis (Figure II.5A) (PCoA1 and PCoA2 explain 33.1% and 23.01% of the variance, respectively) revealed a separation of all samples, weighted analysis clustered the CP21F, G21M and OAZ21F samples together (Figure II.5B) (PCoA1 and PCoA2 explain 79.28% and 12% of the variance, respectively), which is supported by the low dissimilarity coefficients depicted in Figure II.5C. On the other hand, OAZ21M, G21F and LabM samples distance from the remaining and between them (Figure II.5B), also supported by the increased coefficients of dissimilarity (Figure II.5C).

Flower diagram represented in Figure II.6 depicts a total of 34 OTUs shared between all samples. Among these, 12 OTUs (35.3%) corresponded to members of the Acetobacteraceae family (*Acetobacter*, *Gluconobacter* and *Komagataeibacter* genera) (data not shown).

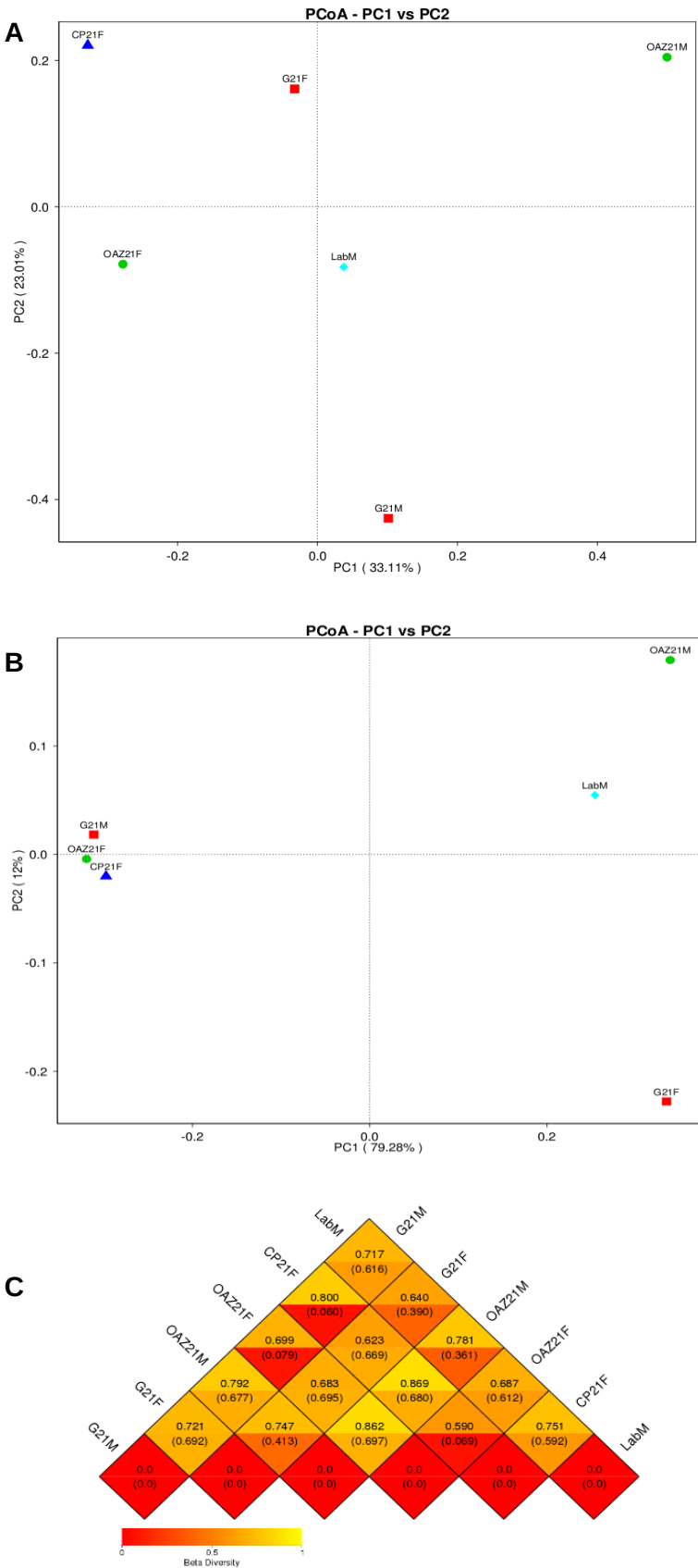


Figure II.5 – Beta-diversity analysis of the microbial communities. Principal Coordinate Analysis (PCoA) regarding the Unweighted (A) and Weighted (B) UniFrac calculations for all samples, displaying the distance between all samples. (C) Pairwise dissimilarity coefficients obtained by Unweighted (top values) and Weighted (bottom values between parentheses) UniFrac measurements.

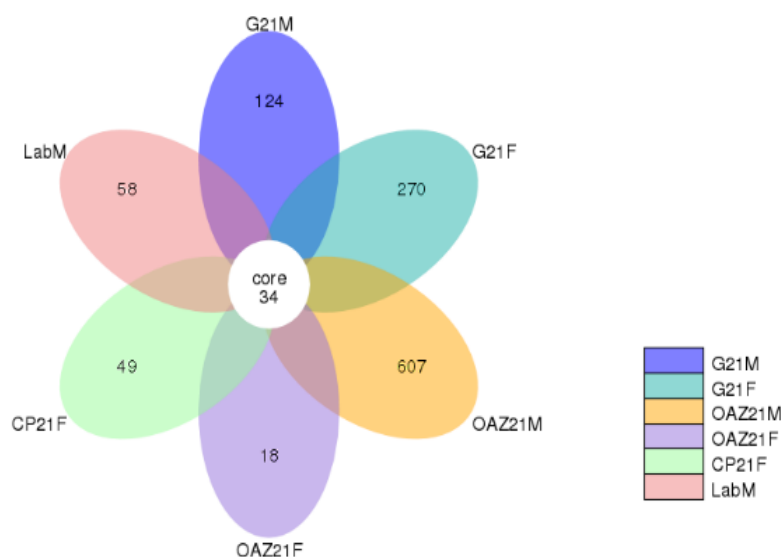


Figure II.6 - Flower diagram of shared and unique OTUs. Represents the unique OTUs of each sample and the core bacterial OTUs common to all samples.

4. Discussion

The role of the gut microbiome in the physiology, metabolism, and homeostasis of animals is undeniable^{6,77,78}. Particularly, *Drosophila* spp. associate with a limited number of bacterial species mainly from the Acetobacteraceae, Enterobacteriaceae and Lactobacillaceae families^{7,61}. These host-microbe associations have gained attention over the past years as a possible strategy, via microbiome manipulation, to control insect pests, such as *D. suzukii*, which still mainly rely on pesticides for its control⁵³, or inefficient cultural measures⁷⁹.

This study aimed to fill the gaps in the knowledge regarding the *D. suzukii* gut-associated bacteria and establish a core microbiome. First observations revealed that the Proteobacteria phylum was the most representative group in all samples, up to 98.8% abundance. This observation is corroborated by previous studies that identified this group as the most prominent in *D. suzukii*^{56,61,64,65}.

The analysis of the bacterial community harboured by female wild-collected *D. suzukii* revealed a dominance of *Komagataeibacter*, *Acetobacter* and *Gluconobacter*. This pattern was observed through most of the samples, apart from OAZ21M and LabM samples, which, nevertheless, still displayed AAB group in their top 10 of most abundant genera. This observation shows a clear and unsurprising dominance by AAB in wild *D. suzukii*. AAB has been thoroughly linked to drosophilids, due to their presence in fermenting environments, which *Drosophila* spp. are rather attracted to^{80,81}.

In *D. melanogaster*, *Acetobacter thailandicus* has been established as a mutualistic symbiont⁶⁶, once more stressing the importance of AAB to *Drosophila*. Regarding *D. suzukii*, Chandler et al (2014) identified a relatively high abundance of AAB in wild samples of adult flies. Moreover, another study conducted by Vacchini *et al* (2017) isolated several species of AAB from *D. suzukii* fed in a fruit-based diet⁶⁰. Furthermore, the core microbiome analysis of *D. suzukii* displayed, in this study, the dominance of Acetobacteraceae. This observation supports, once more, the importance of AAB to *D. suzukii* as it depicts the stability and persistence of this bacterial taxa to associate with the host in different environments, suggesting an obligatory relationship.

Other genera seemed to be associated with the analysed samples, for instance, the *Pseudomonas* genus, which not only was identified as part of the core bacterial community but was the major representative in laboratory-reared *D. suzukii*. This observation contradicts a previous report, which revealed the dominance of Enterobacteriaceae in laboratory-reared flies⁵⁸ and, instead, may suggest a stable association of *Pseudomonas* bacteria with *D. suzukii* as it persists in the gut. This is not the first report of *Pseudomonas* associated with *D. suzukii*, and it must be taken in consideration the vast number of species of this genus with different interactions with the hosts. For example, there have been reports on the detrimental activity of some species of *Pseudomonas* (*P. entomophila* and *P. protegens*)^{82,83}. *Pantoea* was also present relatively abundantly in all samples, also corroborating previous findings^{56,65}.

In terms of diversity and richness of each sample, males developed in raspberry (OAZ21M) presented the higher indices of species richness, supported by its rarefaction curve, which did not saturate. However, the diversity of the microbial community was higher for females developed in blueberry (G21F), as shown by the Shannon and Simpson indices. These results indicate that although males developed in raspberry (OAZ21M) revealed a richer community (Chao1 and ACE indices), the distribution of this richness did not represent as much diversity as the G21F sample. These observations do not distinguish or quantify the influence of sex and diet upon the microbial diversity of the populations. One possible explanation is a synergetic interaction of these two parameters, contributing to the variability of microbiota. However, to analyse only the influence of diet, grouped samples (female and males) of OAZ21 revealed a higher richness than G21 individuals, while diversity was higher in G21. A previous report disclosed a slightly enhanced richness and diversity in the first generation of individuals fed in a blueberry-based diet, when compared to a raspberry-based diet, corroborating our findings on the diversity, but contradicting the richness

indices⁶⁴. However, in this previous study, changes were more significant only after 4 generations, meaning that a longer observation would be required to accurately understand the role of diet in shaping the microbiome. Moreover, to assess the uniqueness of the bacterial communities between the two growing environments, Venn diagram discloses a total of 74.5% of unique OTUs. This supports a divergence in the bacterial community and supports an influence of the diet in shaping the composition of the microbiome of *D. suzukii*.

PCoA was employed to further quantify the distance between the samples. The unweighted UniFrac PCoA revealed no clustering of any of the samples, suggesting that phylogenetically, the observed communities were unrelated, which is supported by the unweighted pairwise dissimilarity indices. This could be explained by the presence of several unique sequences in each sample as it is displayed in the Flower Diagram. On the other hand, the weighted UniFrac PCoA clustered CP21F, OAZ21F and G21M samples together, suggesting that the dominant sequences in the community were phylogenetically close. This observation is, in fact, corroborated by the presence of AAB in very similar proportions in the three samples, once again stressing the ecological association of these bacteria with *D. suzukii*. Contrarily, OAZ21M, G21F and LabM did not correlate with each other in the weighted analysis. Although the dissimilarity coefficients were reduced, the samples did not cluster together, hindering the establishment of any correlation between them. Nevertheless, it may reveal a tendency of OAZ21M and G21F to shift towards a bacterial community similar to that of an artificial-diet population. Even though this suggestion may be valid, the shaping factor remains unclear. The individual influence of sex or diet is undistinguishable according to these results, once again possibly suggesting a interaction of both parameters. Previous reports, as previously mentioned, reported the influence of diet in shaping the microbiome⁶⁴, but the influence of sex has been also studied. Data collected by Bing *et al* (2018) showed no difference in the diversity of bacteria between male and female samples⁵⁸. However, the influence of the microbiome seems to differentiate according to sex, as data published by Shu *et al* (2021) reveals⁸⁴.

Although the isolation of bacteria was not quantitative, we were able to analyse the diversity of bacterial isolates in wild and fruit-emerged *D. suzukii* from different Portuguese orchards. Wild female *D. suzukii* were found to have the most diverse community of isolated bacteria, being represented by 10 different families. However, fruit-emerged *D. suzukii* did not reveal any noticeable difference in diversity between male and female flies. Moreover, by isolating bacteria, the impact of different fruits as a

shaping factor of diversity was not clear. Instead, the location seemed to be the major influence. This has been previously discussed in other studies^{56,65}.

The most prominent genus of isolated bacteria which was found across the majority of collected samples was *Frigoribacterium*. Species from this genus were identified mostly in different soil samples and as root endophytic bacteria^{85–88}. Although this genus has been previously isolated from other insects, such as Hymenoptera, Coleoptera, and Lepidoptera^{89–91}, this is the first report of its identification in *D. suzukii* and *Drosophila* spp. in general. This genus is poorly characterized and little is known to infer any particular properties or explanation for its association with *D. suzukii*. Nevertheless, the identification of this genus across samples from different Portuguese orchards may reveal a specific link with these populations and a possible genotype-specific relationship. To further deepen this hypothesis, the mutualistic association between *D. suzukii* and these isolates of *Frigoribacterium* must be assessed.

Another interesting result was the identification of *Tatumella* in larvae, which corroborates the observations of other studies, that also revealed a prominence of this genus in *D. suzukii* larvae^{56,62}. However, its association with *D. suzukii* may still be controversial as they were reported to decrease lifespan of adult flies⁵⁶. This genus is also particularly interesting as it seems to specifically associate with *D. suzukii* as it was not identified in other *Drosophila* spp. Moreover, Solomon *et al.* (2018) demonstrated that a *Tatumella* isolate from *D. suzukii* did not cause any significant change in the development of monoassociated *D. melanogaster* larvae, which further suggests a species-specific association⁵⁹.

In conclusion, this synergetic approach of bacteriome analysis came to both corroborate previous findings and open new doors to the study of *D. suzukii* microbiome. Even though *D. suzukii* is a highly polyphagous pest, meaning that it probably does not develop associated with a unique host²⁹, in larger productions, there may be a close association with a single crop. For this reason, the study aimed to understand the impact of fruits as a shaping factor of the microbiota and found that both raspberry and blueberry samples distanced significantly from the other populations. The individual effect of diet and/or sex was unquantifiable, and to overcome this, future and larger studies should be performed to understand the true impact of these factors. Nevertheless, we were able to further confirm a close association of *D. suzukii* to AAB in all samples, proposing this taxon as an important member of the *D. suzukii* core microbiome. Moreover, the isolation of *Tatumella* is further corroborating a possible association of this genus with *D. suzukii* larvae and

Frigoribacterium as a possible new genus associated with this pest. This approach filled some gaps and further consolidated information about the *D. suzukii* microbiome in hopes, in the future, to develop microbiome manipulation and natural biocontrol strategies to control this pest.

References

1. Zilber-Rosenberg, I. & Rosenberg, E. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol Rev* **32**, 723–735 (2008).
2. Carthey, A. J. R., Blumstein, D. T., Gallagher, R. v., Tetu, S. G. & Gillings, M. R. Conserving the holobiont. *Funct Ecol* **34**, 764–776 (2019).
3. Schroeder, B. O. & Bäckhed, F. Signals from the gut microbiota to distant organs in physiology and disease. *Nat Med* **22**, 1079–1089 (2016).
4. Sharon, G., Sampson, T. R., Geschwind, D. H. & Mazmanian, S. K. The Central Nervous System and the Gut Microbiome. *Cell* **167**, 915–932 (2016).
5. Ruff, W. E., Greiling, T. M. & Kriegel, M. A. Host–microbiota interactions in immune-mediated diseases. *Nat Rev Microbiol* **18**, 521–538 (2020).
6. Barko, P. C., McMichael, M. A., Swanson, K. S. & Williams, D. A. The Gastrointestinal Microbiome: A Review. *J Vet Intern Med* **32**, 9–25 (2018).
7. Broderick, N. A. & Lemaitre, B. Gut-associated microbes of *Drosophila melanogaster*. *Gut Microbes* **3**, 307–321 (2012).
8. Chandler, J. A., James, P. M., Jospin, G. & Lang, J. M. The bacterial communities of *Drosophila suzukii* collected from undamaged cherries. *PeerJ* **2**, e474 (2014).
9. Engel, P. & Moran, N. A. The gut microbiota of insects-diversity in structure and function. *FEMS Microbiol Rev* **37**, 699–735 (2013).
10. Leech, T. *et al.* Social environment drives sex and age-specific variation in *Drosophila melanogaster* microbiome composition and predicted function. *Mol Ecol* **30**, 5831–5843 (2021).
11. Chandler, J. A., Lang, J., Bhatnagar, S., Eisen, J. A. & Kopp, A. Bacterial communities of diverse *Drosophila* species: ecological context of a host-microbe model system. *PLoS Genet* **7**, e1002272 (2011).
12. Staubach, F., Baines, J. F., Künzel, S., Bik, E. M. & Petrov, D. A. Host Species and Environmental Effects on Bacterial Communities Associated with *Drosophila* in the Laboratory and in the Natural Environment. *PLoS One* **8**, e70749 (2013).
13. Medina, V. *et al.* Characterized non-transient microbiota from stinkbug (*Nezara viridula*) midgut deactivates soybean chemical defenses. *PLoS One* **13**, e0200161 (2018).

14. Staudacher, H. *et al.* Variability of Bacterial Communities in the Moth *Heliothis virescens* Indicates Transient Association with the Host. *PLoS One* **11**, e0154514 (2016).
15. Wang, S. *et al.* An Insight into Diversity and Functionalities of Gut Microbiota in Insects. *Curr Microbiol* **77**, 1976–1986 (2020).
16. Ceja-Navarro, J. A. *et al.* Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. *Nat Commun* **6**, 1–9 (2015).
17. Jing, T. Z., Qi, F. H. & Wang, Z. Y. Most dominant roles of insect gut bacteria: digestion, detoxification, or essential nutrient provision? *Microbiome* **8**, 1–20 (2020).
18. Marasco, R. *et al.* Destabilization of the Bacterial Interactome Identifies Nutrient Restriction-Induced Dysbiosis in Insect Guts. *Microbiol Spectr* **10**, e01580-21 (2022).
19. Mereghetti, V., Chouaia, B. & Montagna, M. New Insights into the Microbiota of Moth Pests. *Int J Mol Sci* **18**, 2450 (2017).
20. Shakeel, M. *et al.* Insect Microbiota and Host Immunity: An Emerging Target for Pest Control. *New and Future Development in Biopesticide Research: Biotechnological Exploration* 261–280 (2022).
21. Gao, X. *et al.* Biodiversity of the microbiota in *Spodoptera exigua* (Lepidoptera: Noctuidae). *J Appl Microbiol* **126**, 1199–1208 (2019).
22. Pittman, G. W., Brumbley, S. M., Allsopp, P. G. & O'Neill, S. L. Assessment of gut bacteria for a paratransgenic approach to control *Dermolepida albobirtum* larvae. *Appl Environ Microbiol* **74**, 4036–4043 (2008).
23. Gao, X. *et al.* Biodiversity of the microbiota in *Spodoptera exigua* (Lepidoptera: Noctuidae). *J Appl Microbiol* **126**, 1199–1208 (2019).
24. Tang, X. *et al.* Complexity and Variability of Gut Commensal Microbiota in Polyphagous Lepidopteran Larvae. *PLoS One* **7**, e36978 (2012).
25. Hadapad, A. B., Shettigar, S. K. G. & Hire, R. S. Bacterial communities in the gut of wild and mass-reared *Zeugodacus cucurbitae* and *Bactrocera dorsalis* revealed by metagenomic sequencing. *BMC Microbiol* **19**, 1–11 (2019).
26. Ajene, I. J. *et al.* Microbiome diversity in *Diaphorina citri* populations from Kenya and Tanzania shows links to China. *PLoS One* **15**, e0235348 (2020).

27. Xia, X. *et al.* Metagenomic Sequencing of Diamondback Moth Gut Microbiome Unveils Key Holobiont Adaptations for Herbivory. *Front Microbiol* **8**, 663 (2017).
28. Lee, J. C. *et al.* The susceptibility of small fruits and cherries to the spotted-wing drosophila, *Drosophila suzukii*. *Pest Manag Sci* **67**, 1358–1367 (2011).
29. Asplen, M. K. *et al.* Invasion biology of spotted wing *Drosophila* (*Drosophila suzukii*): a global perspective and future priorities. *J Pest Sci (2004)* **88**, 469–494 (2015).
30. Kaneshiro KY. *Drosophila* (Sophophora) *suzukii* (Matsumura). *Proc Hawaiian Entomol Soc* **24**, 179 (1983).
31. Kanzawa, T. Studies on *Drosophila suzukii* Mats. *Rev Appl Entomol* **24**, 315 (1936).
32. Hauser M, Gaimari S & Damus M. *Drosophila suzukii* new to North America. *Fly times* **43**, 12–15 (2009).
33. Calabria, G., Máca, J., Bächli, G., Serra, L. & Pascual, M. First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. *Journal of Applied Entomology* **136**, 139–147 (2012).
34. Deprá, M., Poppe, J. L., Schmitz, H. J., de Toni, D. C. & Valente, V. L. S. The first records of the invasive pest *Drosophila suzukii* in the South American continent. *J Pest Sci (2004)* **87**, 379–383 (2014).
35. Santos, L. A. dos *et al.* Global potential distribution of *Drosophila suzukii* (Diptera, Drosophilidae). *PLoS One* **12**, 1–13 (2017).
36. de Ros, G., Grassi, A. & Pantezzi, T. Recent Trends in the Economic Impact of *Drosophila suzukii*. *Drosophila suzukii Management* 11–27 (2020).
37. Bolda, M. P., Goodhue, R. E. & Zalom, F. G. Spotted Wing *Drosophila*: Potential Economic Impact of a Newly Established Pest. *ARE Update* **13**, 5–8 (2010).
38. de Ros, G., Anfora, G., Grassi, A. & Ioriatti, C. The potential economic impact of *Drosophila suzukii* on small fruits production in Trentino (Italy). *Integrated protection of fruit crops IOBC-WPRS Bulletin* **91**, 317–321 (2013).
39. Pimentel, D. Environmental and Economic Costs of the Application of Pesticides Primarily in the United States. *Environ Dev Sustain* **7**, 229–252 (2005).
40. Yeh, D. A., Drummond, F. A., Gómez, M. I. & Fan, X. The Economic Impacts and Management of Spotted Wing *Drosophila* (*Drosophila suzukii*): The Case of Wild Blueberries in Maine. *J Econ Entomol* **113**, 1262–1269 (2020).

41. Mazzi, D., Bravin, E., Meraner, M., Finger, R. & Kuske, S. Economic Impact of the Introduction and Establishment of *Drosophila suzukii* on Sweet Cherry Production in Switzerland. *Insects* **8**, 18 (2017).
42. de Ros, G., Conci, S., Pantezzi, T. & Savini, G. The economic impact of invasive pest *Drosophila suzukii* on berry production in the Province of Trento, Italy. *J Berry Res* **5**, 89–96 (2015).
43. Knapp, L., Mazzi, D. & Finger, R. The economic impact of *Drosophila suzukii*: perceived costs and revenue losses of Swiss cherry, plum and grape growers. *Pest Manag Sci* **77**, 978 (2021).
44. Farnsworth, D. *et al.* Economic analysis of revenue losses and control costs associated with the spotted wing drosophila, *Drosophila suzukii* (Matsumura), in the California raspberry industry. *Pest Manag Sci* **73**, 1083–1090 (2017).
45. van Timmeren, S. & Isaacs, R. Control of spotted wing drosophila, *Drosophila suzukii*, by specific insecticides and by conventional and organic crop protection programs. *Crop Protection* **54**, 126–133 (2013).
46. Bruck, D. J. *et al.* Laboratory and field comparisons of insecticides to reduce infestation of *Drosophila suzukii* in berry crops. *Pest Manag Sci* **67**, 1375–1385 (2011).
47. Spies, J. M. & Liburd, O. E. Comparison of Attractants, Insecticides, and Mass Trapping for Managing *Drosophila suzukii* (Diptera: Drosophilidae) in Blueberries. *Florida Entomologist* **102**, 315–321 (2019).
48. Sookhtanlou, M., Allahyari, M. S. & Surujlal, J. Health Risk of Potato Farmers Exposed to Overuse of Chemical Pesticides in Iran. *Saf Health Work* **13**, 23–31 (2022).
49. Haviland, D. R. & Beers, E. H. Chemical Control Programs for *Drosophila suzukii* that Comply With International Limitations on Pesticide Residues for Exported Sweet Cherries. *J Integr Pest Manag* **3**, 1–6 (2012).
50. Gress, B. E. & Zalom, F. G. Identification and risk assessment of spinosad resistance in a California population of *Drosophila suzukii*. *Pest Manag Sci* **75**, 1270–1276 (2019).
51. Disi, J. O. & Sial, A. A. Laboratory Selection and Assessment of Resistance Risk in *Drosophila suzukii* (Diptera: Drosophilidae) to Spinosad and Malathion. *Insects* **12**, 794 (2021).
52. Smirle, M. J., Zurowski, C. L., Ayyanath, M. M., Scott, I. M. & MacKenzie, K. E. Laboratory studies of insecticide efficacy and resistance in *Drosophila suzukii*

- (Matsumura) (Diptera: Drosophilidae) populations from British Columbia, Canada. *Pest Manag Sci* **73**, 130–137 (2017).
53. Wang, X. Biological control of *Drosophila suzukii*. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* **15**, (2020).
 54. Cossentine, J., Robertson, M. & Xu, D. Biological Activity of *Bacillus thuringiensis* in *Drosophila suzukii* (Diptera: Drosophilidae). *J Econ Entomol* **109**, 1071–1078 (2016).
 55. Bing, X. L., Winkler, J., Gerlach, J., Loeb, G. & Buchon, N. Identification of natural pathogens from wild *Drosophila suzukii*. *Pest Manag Sci* **77**, 1594–1606 (2021).
 56. Hiebert, N., Carrau, T., Bartling, M., Vilcinskas, A. & Lee, K. Z. Identification of entomopathogenic bacteria associated with the invasive pest *Drosophila suzukii* in infested areas of Germany. *J Invertebr Pathol* **173**, 1–11 (2020).
 57. Hiebert, N. *et al.* The Gram-Positive Bacterium *Leuconostoc pseudomesenteroides* Shows Insecticidal Activity against Drosophilid and Aphid Pests. *Insects* **11**, 1–15 (2020).
 58. Bing, X., Gerlach, J., Loeb, G. & Buchon, N. Nutrient-Dependent Impact of Microbes on *Drosophila suzukii* Development. *mBio* **9**, e02199-17 (2018).
 59. Solomon, G. M., Dodangoda, H., McCarthy-Walker, T. T., Ntim-Gyakari, R. R. & Newell, P. D. The microbiota of *Drosophila suzukii* influences the larval development of *Drosophila melanogaster*. *PeerJ* **7**, e8097 (2019).
 60. Vacchini, V. *et al.* Bacterial diversity shift determined by different diets in the gut of the spotted wing fly *Drosophila suzukii* is primarily reflected on acetic acid bacteria. *Environ Microbiol Rep* **9**, 91–103 (2017).
 61. Lin, Q. *et al.* Analyses of the gut bacteriomes of four important *Drosophila* pests. *Canadian Entomologist* **153**, 757–773 (2021).
 62. Chandler, J. A., James, P. M., Jospin, G. & Lang, J. M. The bacterial communities of *Drosophila suzukii* collected from undamaged cherries. *PeerJ* **2**, e474 (2014).
 63. Fountain, M. T. *et al.* Alimentary microbes of winter-form *Drosophila suzukii*. *Insect Mol Biol* **27**, 383–392 (2018).
 64. Jiménez-Padilla, Y., Esan, E. O., Floate, K. D. & Sinclair, B. J. Persistence of diet effects on the microbiota of *Drosophila suzukii* (Diptera: Drosophilidae). *Canadian Entomologist* **152**, 516–531 (2020).

65. Martinez-Sañudo, I. *et al.* Metagenomic analysis reveals changes of the *Drosophila suzukii* microbiota in the newly colonized regions. *Insect Sci* **25**, 833–846 (2018).
66. Pais, I. S., Valente, R. S., Sporniak, M. & Teixeira, L. *Drosophila melanogaster* establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. *PLoS Biol* **16**, e2005710 (2018).
67. van Timmeren, S., Diepenbrock, L. M., Bertone, M. A., Burrack, H. J. & Isaacs, R. A Filter Method for Improved Monitoring of *Drosophila suzukii* (Diptera: Drosophilidae) Larvae in Fruit. *J Integr Pest Manag* **8**, 23 (2017).
68. Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697 (1991).
69. Magoč, T. & Salzberg, S. L. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27**, 2957–2963 (2011).
70. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**, 335 (2010).
71. Quast, C. *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41**, (2013).
72. Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194–2200 (2011).
73. Edgar, R. C. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* **10**, 996–998 (2013).
74. R Core Team. R: A Language and Environment for Statistical Computing. Preprint at <https://www.R-project.org/> (2020).
75. Zhang, B. & Horvath, S. A general framework for weighted gene co-expression network analysis. *Stat Appl Genet Mol Biol* **4**, (2005).
76. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (Springer-Verlag New York, 2016).
77. Turnbaugh, P. J. *et al.* The Human Microbiome Project. *Nature* **449**, 804–810 (2007).
78. Clemmons, B. A., Voy, B. H. & Myer, P. R. Altering the Gut Microbiome of Cattle: Considerations of Host-Microbiome Interactions for Persistent Microbiome Manipulation. *Microb Ecol* **77**, 523–536 (2019).

79. Schöneberg, T. *et al.* Cultural Control of *Drosophila suzukii* in Small Fruit—Current and Pending Tactics in the U.S. *Insects* **12**, 172 (2021).
80. Crotti, E. *et al.* Acetic acid bacteria, newly emerging symbionts of insects. *Appl Environ Microbiol* **76**, 6963–6970 (2010).
81. Mazzetto, F. *et al.* Olfactory attraction of *Drosophila suzukii* by symbiotic acetic acid bacteria. *J Pest Sci (2004)* **89**, 783–792 (2016).
82. Choudry, B., Durance, A., Ibtisam, A. & Kim, T. Determination of the toxicity of *Pseudomonas protegens* CHA0 to *Drosophila suzukii* (Spotted Winged *Drosophila*) larvae through oral inoculation. *Undergraduate Journal of Experimental Microbiology and Immunology* **8**, 1–6 (2022).
83. Carrau, T. *et al.* The Cellular Innate Immune Response of the Invasive Pest Insect *Drosophila suzukii* against *Pseudomonas entomophila* Involves the Release of Extracellular Traps. *Cells* **10**, 3320 (2021).
84. Shu, R. *et al.* Sex-Dependent Effects of the Microbiome on Foraging and Locomotion in *Drosophila suzukii*. *Front Microbiol* **12**, 656406 (2021).
85. Kong, D. *et al.* *Frigoribacterium salinisoli* sp. Nov., isolated from saline soil, transfer of *Frigoribacterium mesophilum* to *Parafrigoribacterium* gen. nov. as *Parafrigoribacterium mesophilum* comb. nov. *Int J Syst Evol Microbiol* **66**, 5252–5259 (2016).
86. Dastager, S. G., Lee, J. C., Ju, Y. J., Park, D. J. & Kim, C. J. *Frigoribacterium mesophilum* sp. nov., a mesophilic actinobacterium isolated from Bigeum Island, Korea. *Int J Syst Evol Microbiol* **58**, 1869–1872 (2008).
87. Kämpfer, P. *et al.* *Frigoribacterium faeni* gen. nov., sp. nov., a novel psychrophilic genus of the family Microbacteriaceae. *Int J Syst Evol Microbiol* **50**, 355–363 (2000).
88. Wang, H. F. *et al.* *Frigoribacterium endophyticum* sp. nov., an endophytic actinobacterium isolated from the root of *Anabasis elatior* (C. A. Mey.) Schischk. *Int J Syst Evol Microbiol* **65**, 1207–1212 (2015).
89. Khan, S., Somerville, D., Frese, M. & Nayudu, M. Environmental gut bacteria in European honey bees (*Apis mellifera*) from Australia and their relationship to the chalkbrood disease. *PLoS One* **15**, e0238252 (2020).
90. He, C., Nan, X., Zhang, Z. & Li, M. Composition and diversity analysis of the gut bacterial community of the oriental armyworm, *Mythimna separata*, determined by

culture-independent and culture-dependent techniques. *Journal of Insect Science* **13**, 165 (2013).

91. Adams, A. S., Boone, C. K., Bohlmann, J. & Raffa, K. F. Responses of Bark Beetle-Associated Bacteria to Host Monoterpenes and Their Relationship to Insect Life Histories. *J Chem Ecol* **37**, 808–817 (2011).

Supplementary Materials

Table SII-6 - Samples of *D. suzukii* used for identification of bacterial communities by isolation and metagenomics.

Samples	Abbreviation	Isolation	Metagenomic
Castelo de Paiva, 2021 (Females)	CP21F	Yes	Yes
Castelo de Paiva, 2021 (Males)	CP21M	Yes	DNA with not enough quality for sequencing
Castelo de Paiva, 2021 (Larva)	CP21L	Yes	Not enough sample
Vila Nova de Gaia 2021 (Females)	G21F	Yes	Yes
Vila Nova de Gaia 2021 (Males)	G21M	Yes	Yes
Oliveira de Azeméis 2021 (Females)	OAZ21F	Yes	Yes
Oliveira de Azeméis 2021 (Males)	OAZ21M	Yes	Yes
Santa Maria da Feira 2021 (Females)	SMF21F	Yes	Not enough sample
Santa Maria da Feira 2021 (Males)	SMF21M	Yes	Not enough sample
Laboratory (Females)	LabF	Yes	DNA with not enough quality for sequencing
Laboratory (Males)	LabM	Yes	Yes

III. Chapter 3: *Drosophila suzukii* displays a sex-dependent immune response to *Microbacterium thalassium* and *Providencia* sp. infection

Abstract

Drosophila suzukii is an invasive plant pest whose consequences to producers demand the application of efficient control measures. To mitigate the use of pesticides, which remain the main control method, research is now focused on developing biocontrol tools, such as the search for natural entomopathogens of *D. suzukii*. The goal of this study was to assess if *D. suzukii* flies infected with two new putative entomopathogenic bacteria (*Microbacterium thalassium* and *Providencia* sp.) showed disturbs in their lifespan and/or in the fly's immunity molecular responses. Female and male flies were fed with one of these bacteria, and the lifespan was monitored for six days after infection. Expression of genes involved in the immune deficiency (Imd) and the Toll pathways was assessed at 6 and 24 hours of exposure. Despite the lifespan not being significantly disturbed, there was an upregulation of the Dual-oxidase gene (*duox*), involved in ROS-dependent regulation of gut bacteria and late activation of the Toll pathway. Moreover, sex influenced the immune molecular responses as females' infection led to an earlier expression of AMPs (diptericin) and were more susceptible to *Providencia* sp., while male flies were more susceptibility to *M. thalassium* whose infection induced the expression of genes predominantly associated with the Toll signalling pathway. These data demonstrate some immunological pathways involved in the response of SWD to these bacteria and that the immune response dynamics is sex-dependent.

1. Introduction

Drosophila suzukii is an important plant pest with a current worldwide distribution^{1,2} with severe economic consequences for producers of soft-skinned fruits and berries^{3–10}. The aggravation of this pest demands a heightened use of pesticides, which remains the go-to strategy, leading to undesired side-effects ranging from ecological damage, public health hazards, and pesticide resistance^{11–16}. There is, therefore, an urgent need for more effective and safer alternatives, to comply with integrated pest management programs (IPM)¹⁷.

Management programs started to move towards alternative strategies that rely on the biological control of pests^{18–20} and the search for new biological control agents has become inevitable²¹. The use of generalist entomopathogenic bacteria, such as *Bacillus thuringiensis* has been proven to be an efficient pest control agent against a range of insect pests^{22–24}. However, broad-spectrum activity and potential development of pest resistance to *B. thuringiensis* toxins²⁵ require the identification of naturally occurring entomopathogens of plant pests²⁶.

Studies have already dwelled on the identification of natural pathogens of *D. suzukii*^{27–29}. However, the combination of efficiency and safety reveals to be a challenging task as some of the identified pathogenic species are also human pathogens (e.g. *Alcaligenes faecalis* and *Achromobacter spanius*)^{29–31}. Among the identified species, *Leuconostoc pseudomesenteroides* has been proven to decrease the survival of *D. suzukii*^{27,28} and no reports of human pathogenicity have been published; in fact, the probiotic activity of this bacteria has been reported³², encouraging the establishment of this species as a new microbial control agent for this pest.

Species from the *Providencia* genus (*P. rettgeri*, *P. sneebia*, *P. alcalifaciens*, *P. burhodogranariae*) reported insecticidal activity towards *D. melanogaster* and are, therefore, good candidates to test their pathogenicity towards *D. suzukii*³³. Moreover, *P. vermicola* is a particular species isolated from entomopathogenic nematodes and, thus, a possible candidate for biological control^{34,35}. *Microbacterium* spp. have been isolated from other plant pests and disclosed insecticidal activity towards *Hermetia illucens* (Diptera: Stratiomyidae), a non-pest insect, and *Ostrinia nubilalis* (Lepidoptera: Pyralidae), a maize pest^{36,37}.

Although insecticidal activity is an important factor for the screening of new entomopathogenic agents, the molecular characterization of immunity pathways may provide important dynamics and mechanisms underlying either sensitivity or resistance to these microorganisms^{38–40}. Hiebert *et al* (2020) have previously conducted a characterization of *D. suzukii* immune response upon infection with different bacteria, revealing a possible overcome of the natural immunity pathways by *L. pseudomesenteroides*, by blocking the production of antimicrobial peptides (AMPs) and leading to an increase of gut tissue damage²⁷. However, this study only focused on the JAK/STAT pathway, which is linked to cell proliferation, and response to tissue damage and external stress⁴¹. Garriga *et al* (2020) conducted another study on the humoral and cellular immune response of *D. suzukii* upon infection with a nematode-bacterial complex, describing the capacity of the bacteria to hinder the production and activity of

AMPs and other defence mechanisms, such as the prophenoloxidase system and phagocytosis⁴². To further deepen the knowledge of the innate immune response of *D. suzukii* to oral infection by putative pathogenic organisms, other specialized immunity pathways must be studied.

Drosophila melanogaster, as a widely studied biological model, has been used for the study of immunity pathways due to their resemblance and homology with human immunity pathways⁴³. Thus, these pathways have been fairly well characterized for a long time, alongside their target genes⁴⁴. The immune deficiency (Imd) and Toll pathways are of great importance when discussing *Drosophila* immunity as they constitute the first line of response against bacterial, fungal, and viral infections⁴⁵.

The Imd pathway, although ubiquitously expressed in *Drosophila* tissues, is linked to the humoral immune response and activation of expression of several AMPs (e.g. dipterecin) in a tissue-specific way along the gut epithelia⁴⁶. The activation of this pathway requires peptidoglycan recognition proteins (PGRPs)^{47,48}, which induce immune responses towards pathogenic bacteria in the gut, especially Gram-negative bacteria⁴⁹. The receptor elicits the activation of Imd protein, leading to the activation of an NF- κ B protein (Relish) by phosphorylation and proteolytic cleavage⁵⁰. The N-terminal portion of Relish is translocated to the nucleus, inducing the gene expression of AMPs and regulators of the Imd pathway^{51,52}.

Moreover, the dual oxidase (Duox) also plays an important role in the regulation of gut bacteria and homeostasis in *Drosophila* by the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent production of reactive oxygen species (ROS) into the gut lumen⁵³. This enzyme is expressed via both Imd-dependent and independent pathways that stimulate Mekk1⁵⁴, a mitogen-activated protein kinase kinase kinase (MAPKKK), which in turn activates p38, an important MAPK in stress response^{55–57}. The subsequent phosphorylation of the activating transcription factor-2 (Atf2) by p38 allows the expression of Duox⁵⁴.

The Toll pathway, similarly to the Imd pathway, also elicits the expression of AMPs (e.g. drosomycin) and is tightly associated with an immune response to Gram-positive bacteria and fungi, requiring PGRP-SA for sensing and trigger of the pathway^{58,59}. The receptors activate the Spätzle-processing enzyme, required for the cleavage of pro-Spätzle into Spätzle, a ligand of the Toll receptor⁶⁰. This pathway culminates in the activation of Dorsal and Dorsal-related immunity (Dif) factor, two transcription factors (TF) involved in the expression of developmental patterning factors (exclusive to Dorsal) and AMPs^{59,61}.

This study aims to unveil the impact of two putative entomopathogenic bacteria, *Providencia* sp. and *Microbacterium thalassium* (previously isolated from *D. suzukii*) on the host's molecular activation of immune pathways. This was evaluated through transcripts expression of genes associated with the Imd and Toll pathways to trace patterns of resistance and sensitivity upon the infection with these microorganisms, according to sex.

2. Materials and Methods

2.1. *D. suzukii* Collection and Rearing

Adult flies of *D. suzukii* were captured from Castelo de Paiva, Portugal (41°03'14.3"N 8°16'49.2"W) (October 2021) and infested fruits with larvae from Santa Maria da Feira, Portugal (40°55'27.4"N 8°33'16.6"W) (October 2021) were kept in sealed transparent boxes at room temperature until *D. suzukii* adults emerged. A pre-established *D. suzukii* laboratory colony was used to perform the experiments and was reared in artificial fly medium (10% sucrose, 8% cornmeal, 4% yeast, 0.08% methyl 4-hydroxybenzoate, 0.15% orthophosphoric acid and 1.5% agar in distilled water) and, which otherwise stated was used throughout the study, and kept at 24°C.

2.2. Sample Processing for Bacterial Isolation

Bacteria were isolated from either captured wild flies or from the first generation of *D. suzukii* adults emerging from the infested fruits. Flies were surface sterilized with 10% bleach for 10 min, followed by 70% EtOH for 5 min, and washed with sterile distilled water. Abdomens were dissected and mashed in sterile phosphate-buffered saline (PBS) pH 7.3 and plated on sterilized Lysogeny Broth (LB) medium (Liofilchem®, Roseto degli Abruzzi, Italy) (1.5% agar), Brain Heart Infusion (BHI) (Merck, Darmstadt, Germany) (1.5% agar) and De Man, Rogosa and Sharpe (MRS) (Panreac AppliChem, Barcelona, Spain) (1.5% agar) and incubated at 25°C for up to 4 days. A single colony from each different morphotype was then isolated.

2.3. Identification of Bacteria

To isolate genomic DNA, a single colony was inoculated into LB and incubated at 25°C at 180 rpm in an orbital agitator overnight. The bacterial suspension was then centrifuged at 5000 *g* for 3 min and the pellet was resuspended in the lysis solution from the GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit (EURx, Gdansk, Poland). Next, DNA purification was performed according to the manufacturer's instructions. DNA samples were amplified through PCR for the partial 16S ribosomal RNA (rRNA) gene using universal primers 27F (5' -

GAGAGTTTGATCCTGGCTCAG - 3') and 1492R (5' – AGAGTTTGATCCTGGCTCAG - 3'), resorting to the following thermal cycler program (95°C for 5 minutes; 24 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 2 minutes; 72°C for 10 minutes). PCR products were then run in an agarose gel (1%) at 120V for 30 minutes, and the ≈1500 bp band was cut from the gel for each sample and purified using the NZYGelpure (NZYTech, Lisbon, Portugal). Sanger sequencing of the purified product was then outsourced at STABVida (Almada, Portugal).

2.4. Oral Infection

Laboratory reared *D. suzukii* flies were orally infected with two different isolates: *M. thalassium* and an isolate belonging to the *Providencia* genus (16S rRNA identity: *P. rettgeri* (100%) and *P. vermicola* (100%)), isolated, respectively, from *D. suzukii* samples from Santa Maria da Feira, Portugal (2021) and Castelo de Paiva, Portugal (2021). 0 to 3 days old flies were collected and aged for 2 days. These aged flies were then transferred to empty narrow vials with a sterile filter paper embedded in tap water overnight to induce starvation and guarantee a uniform feeding for all flies. One day before the infection, bacterial suspensions were also prepared in Mannitol (5% D-mannitol, 0.3% peptone, 0.5% yeast extract) and incubated at 30°C, 180 rpm. Prior the infection, the bacterial suspension was collected by centrifugation at 2000 *g* for 5 min and resuspended to obtain an overconcentrated suspension between ≈10¹⁰ and ≈10¹¹ CFU/ml in a 2.5% sucrose solution, to mimic feeding from bacterial growth in natural environment²⁹. Starved flies were separated by sex, and transferred to another sterilized vial (with 70% EtOH and UV). This vial had a sterile filter paper embedded in the bacterial solution on top of sterile fly medium (without methyl 4-hydroxybenzoate and orthophosphoric acid). Individuals were incubated at 24°C, for 6 and 24 hours.

2.5. Collection of Orally Infected Flies and Colony Forming Units (CFU) Count

To ensure that oral infection was successful and to compare the level of infection at 6 and 24 hours of exposure, all flies from each condition were collected to cold microtubes. Two flies were then transferred to another microtube with 100% cold EtOH to surface sterilize them, while the remaining flies were quickly frozen in liquid nitrogen and stored at -80°C for later assays. The collected flies were homogenized in 100 µL of Mannitol in a bead beater tube with 2.4 mm ceramic beads at 2.4 m/s for 1 min, to release the total bacterial content into the suspension. The suspension was serially diluted and plated in Mannitol agar (1.5% agar) and incubated at 25°C overnight for CFU/fly count.

2.6. Survival Assays

Laboratory reared *D. suzukii* were orally infected with the two isolates previously used, as described in Section 2.4. for 24h and then transferred to sterile fly medium (without methyl 4-hydroxybenzoate and orthophosphoric acid) in 70% EtOH- and UV-sterilized vials. Flies were flipped every two days and mortality was tracked for 6 days. Excessive bacterial growth on the fly food medium impaired the reliable measurement of mortality due to the reingestion of bacteria and the formation of biofilm on top of the fly medium.

2.7. RNA Extraction and cDNA synthesis

Two flies from each condition were homogenized in NZYol (NZYTech, Lisbon, Portugal) and total RNA content was purified according to the manufacturer's recommended protocol, using chloroform purification. Treatment with DNase I (NZYTech, Lisbon, Portugal) was performed before RNA precipitation with isopropanol. RNA was quantified using a microdrop (BMG, Germany) and the integrity of the nucleic acid was verified by running the samples on an agarose gel (1%). To synthesize cDNA from 100 ng of RNA, First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) was used. Samples were incubated at 25°C for 10 min, 50°C for 30 min, and followed by inactivation at 85°C for 5 min. The cDNA was then cooled to 4°C and 1 µl of NZY RNase H (*E. coli*), provided in the same kit, was added to eliminate the remaining RNA. Samples were then incubated at 37°C for 20 min and single-stranded cDNA concentration was measured by resorting to a microdrop (BMG, Germany).

2.8. Gene Expression by Real-Time Quantitative PCR (RT-qPCR)

To analyse the gene expression, 100 ng of each cDNA were added to NZYSupreme qPCR Green Master Mix (NZYTech, Lisbon, Portugal) according to recommendations from the manufacturer. Primers for each tested gene were designed using Primer3Plus⁶² based on the information obtained from the SpottedWingFlyBase (<http://spottedwingflybase.org/>) (*Drosophila suzukii* v1 assembly genome) and NCBI database (<https://www.ncbi.nlm.nih.gov/>) (LBDM_Dsuz_2.1.pri genome). Each primer used for gene expression is listed in Table III-1. RT-qPCR was carried in CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., California, USA) under the following program: 95°C for 2 min, 44 cycles of 95°C for 5 seconds, and 60°C for 30 seconds, and final gradient to obtain melting curve. Reactions were prepared in technical triplicates for each biological replicate. Relative expression was calculated using $2^{-\Delta\Delta C_t}$ after normalization to two housekeeping genes (*Ef1a48D* and *argK*)⁶³.

Table III-1 - Primers used for transcripts expression analysis by RT-qPCR

Gene		5' -> 3' Sequence	GC (%)	Tm (°C)	Product Size (bp)	Source
<i>imd</i>	F	AACGCCCAAACGAACAAC	50	57	100	This study
	R	TGTAGACGGAGCCGAAGTG	58	60		
<i>mekk1</i>	F	CCGTGAGCCGAGTTTGATC	58	59	79	This study
	R	AGCCTTGGCACATCTTGC	56	59		
<i>spz</i>	F	CTTTCCGCAGCATTATCG	50	54	114	This study
	R	GATCTTAAAGGACCCCTG	50	52		
<i>atf2</i>	F	ACGCGCCTCATCAAGAAC	56	58	105	This study
	R	GCTGACATTCTGCTCTGC	56	57		
<i>dif</i>	F	GCTCATTACCCGCAGCAAC	58	60	75	This study
	R	GAACGATTGGATGGGTGC	56	57		
<i>dptA</i>	F	TCCCGATTTCGACGTTG	53	55	83	This study
	R	CCTGTTTTGGGCTTCCAC	56	57		
<i>drs</i>	F	TGTGCCGTCTGGGACAATG	58	61	97	This study
	R	CTTCGCACCAGCACTTCAG	58	59		
<i>duox</i>	F	AATGCCACCGAACTGGAAC	53	58	109	This study
	R	GGATGGGCACAAATCCAAG	53	57		
<i>argK^a</i>	F	CTACCACAACGATGCCAAGA	50	57	180	Zhai <i>et al.</i> (2014)
	R	AAGGTCAGGAAGCCGAGA	56	58		
<i>Ef1α48D^a</i>	F	TGGGCAAGGAAAAGATTAC	45	55	195	Toxopeus <i>et al.</i> (2016)
	R	CGGCCTTCAACTTATCCAAA	45	55		

^a Housekeeping genes used for normalization.

2.9. Statistical Analysis

Statistical analysis was performed using GraphPad Prism V9.0 (GraphPad Software, San Diego, California, USA). Analysis of bacteria levels and relative gene expression were both analysed using Two-Way ANOVA using time of exposure and bacteria as factors and multiple comparisons was adjusted for Tukey's test. Significant differences were considered when $p < 0.05$. Principal Component Analysis (PCA) was employed to disclose correlation between exposure to bacteria and time with relative expression of each gene, separately for females and males.

3. Results

3.1. Bacterial Levels Are Similar at 6 and 24 Hours of Exposure

For both female (Figure III.1A) and male *D. suzukii* flies (Figure III.1B), oral infection was proven to have occurred for *M. thalassium* and *Providencia* sp., as levels significantly ($p < 0.05$) increased in both 6 and 24 hours in comparison to the unexposed flies in a log10 fold-change between 2 CFU/fly and 5.5 CFU/fly. Moreover, exposure time did not reveal any significant impact on bacterial levels in both sexes for every condition. However, although non-significant ($p > 0.05$), male flies presented lower bacterial levels of *M. thalassium* at both 6 and 24 hours when compared to male flies, with a log10 fold increase between 0.9 CFU/fly and 1.7 CFU/fly.

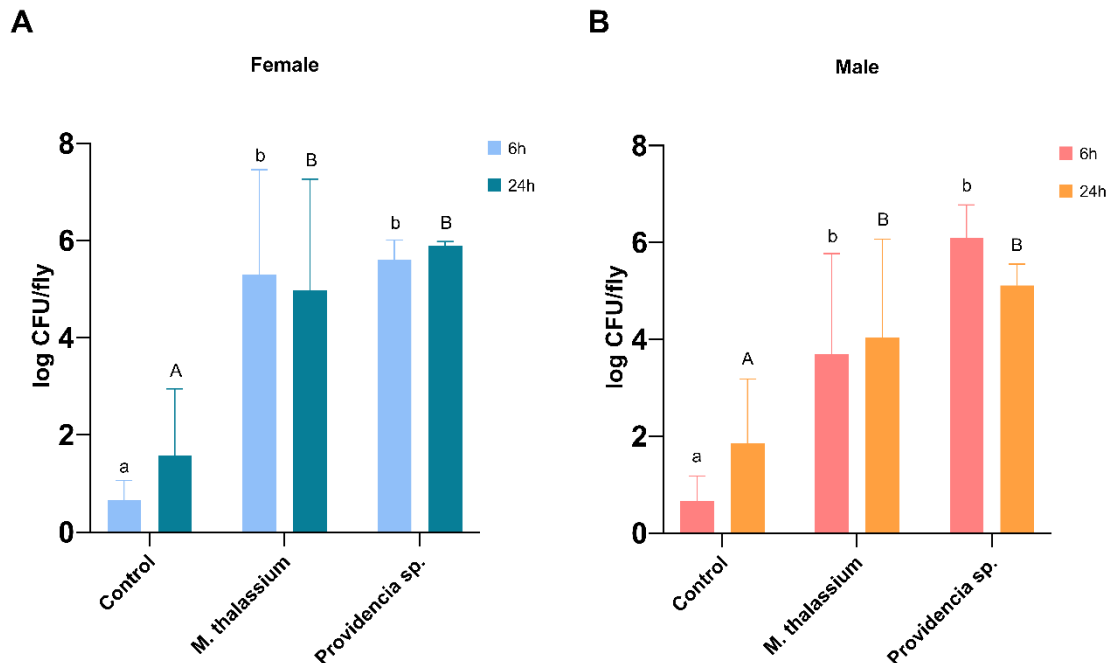


Figure III.1 - Bacterial levels (log CFU/fly) after 6 and 24 hours of exposure. Mean log CFU/fly after 6 and 24 hours of exposure (color coded) to *M. thalassium* and *Providencia* sp. in female (A) and male (B) flies. Different lowercase letters represent significant differences ($p < 0.05$) between conditions in 6 hours of exposure and different uppercase letters represent significant differences ($p < 0.05$) between conditions in 24 hours of exposure.

3.2. Non-Lethal Bacteria Affect *duox* Expression

Although no detectable effect on survival was noted for either bacterium in the course of 6 days (data not shown), immune molecular response was assessed through RT-qPCR to calculate differential relative gene expression of Imd and Toll pathways target genes *duox*, *dptA* and *drs* that encode for the Dual-oxidase, and the AMPs Diptericin A and Drosomycin, respectively. Even though no significant changes in expression were found during the posthoc analysis, ANOVA analysis revealed that exposure time

significantly ($p < 0.01$) contributed to the variation of the expression of *duox* in females, depicted in the increased levels of this gene at 24 hours of exposure to *M. thalassium* (3.0-fold) and *Providencia* sp. (2.2-fold) (Figure III.2A). Contrarily, in males, the condition factor was the main source of variation ($p < 0.05$) at *M. thalassium*, since it prompted increased levels of *duox* at 6 hours (4.4-fold) and 24 hours of exposure (2.7-fold) (Figure III.2B).

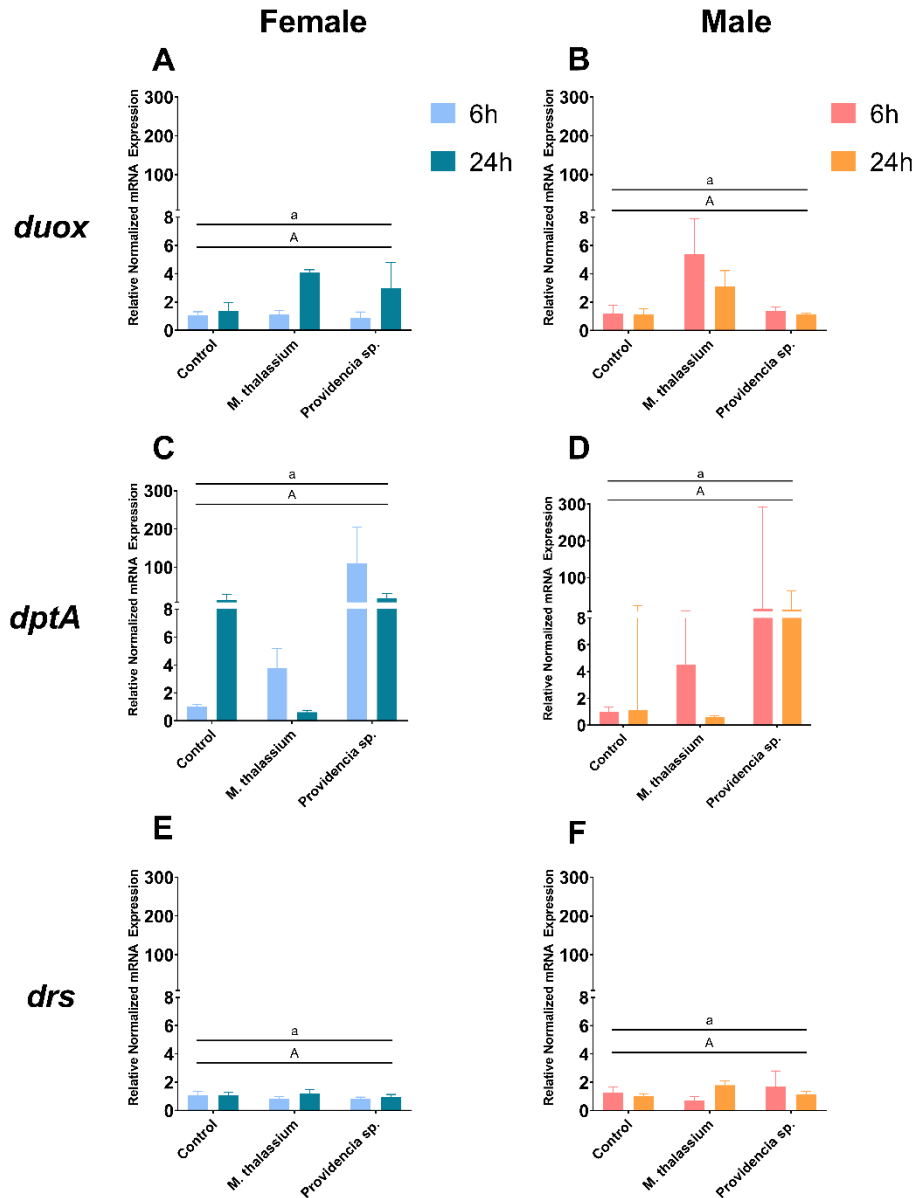


Figure III.2 - Expression of Imd and Toll pathways target genes. Relative normalized mRNA expression of *duox* (A, B), *dptA* (C, D) and *drs* (E, F) in female (A, C, E) and male flies (B, D, F). Error bars represent SEM of means. Different lowercase letters represent significant differences ($p < 0.05$) between conditions in 6 hours of exposure and different uppercase letters represent significant differences ($p < 0.05$) between conditions in 24 hours of exposure and * represents significant differences ($p < 0.05$) between 6 and 24 hours on the same condition.

3.3. *atf2* is Differentially Regulated According to Exposure Time

Besides analysing the gene expression of target genes, the expression of Imd and Toll pathway components was also assessed. The relative expression of two TFs *atf2* and *dif* was calculated and analysed, disclosing no significant ($p > 0.05$) differential expression during post-hoc analysis. However, time of exposure was significantly ($p < 0.05$) associated with variation of *atf2* expression in both females and males. In females, 6 hours of infection with both *M. thalassium* and *Providencia* sp. caused a 1.1 and 1.7-fold decrease in levels of *atf2*, followed by a 1.6 and 1.3-fold increase at 24 hours, respectively (Figure 3A). Contrarily, in males, 6 hours of infection with *M. thalassium* caused a 1.4-fold increase, while *Providencia* sp. did not affect expression. In turn, 24 hours of exposure caused a 1.4-fold decrease in *atf2* levels at 24 hours to both bacteria (Figure III.3B). For *dif*, there was no recognized pattern (Figure III.3C and III.3D).

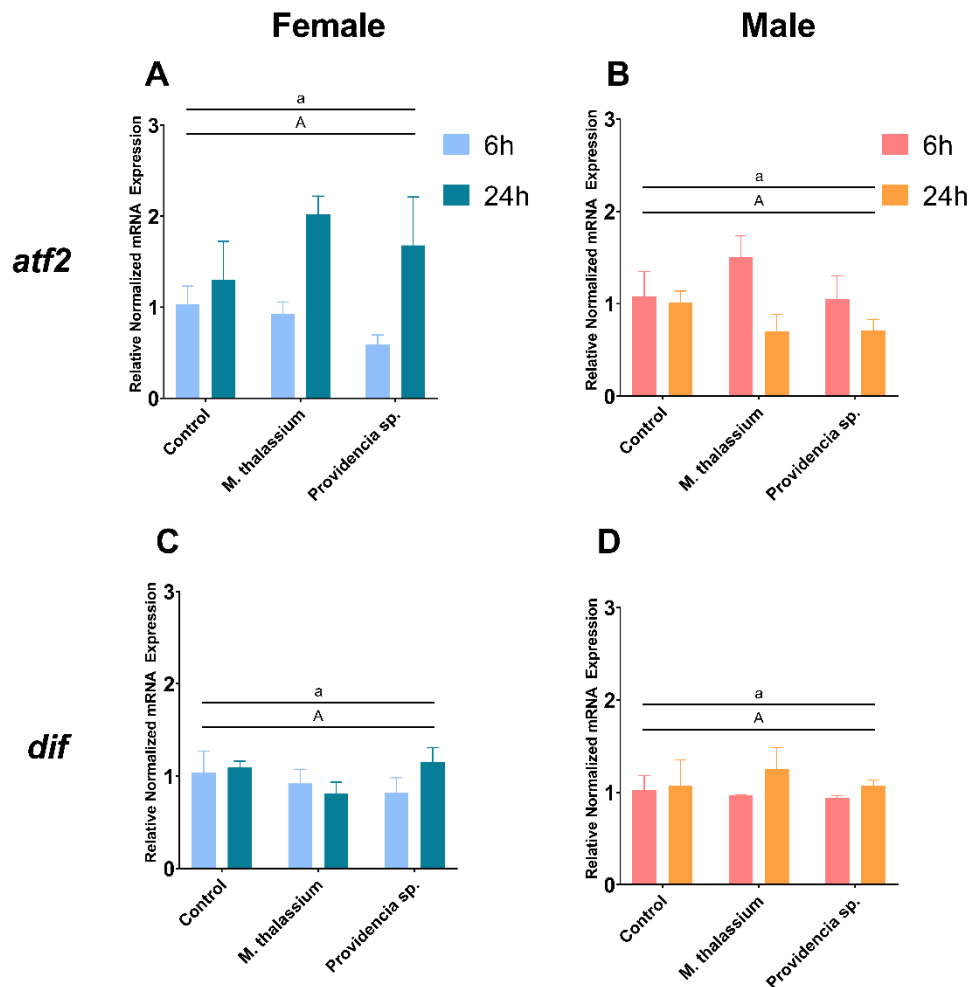


Figure III.3 - Expression of Imd and Toll pathways transcription factors. Relative normalized mRNA expression of *atf2* (A, B) and *dif* (C, D) in female (A, C) and male flies (B, D). Error bars represent SEM of means. Different lowercase

letters represent significant differences ($p < 0.05$) between conditions in 6 hours of exposure and different uppercase letters represent significant differences ($p < 0.05$) between conditions in 24 hours of exposure and * represents significant differences ($p < 0.05$) between 6 and 24 hours on the same condition.

3.4. Females Disclose an Immune Response to *Providencia* sp. and Males to *Microbacterium* sp.

To obtain a broader analysis of the expression of each pathway, one component of each signalling pathway was analysed, namely, *mekk1*, *imd* and *spz*. The expression of *imd* and *spz* was significantly ($p < 0.05$) upregulated from 6 to 24 hours of exposure to *Providencia* sp. in female flies (Figure III.4C and III.4E). Besides revealing a significant ($p < 0.05$) difference in response between *M. thalassium* and *Providencia* sp. in males, according to ANOVA analysis, *spz* was also differentially regulated according to time of exposure, revealing a significant ($p < 0.05$) upregulation of this gene 24 hours after exposure to *M. thalassium*, as depicted in Figure 3F.

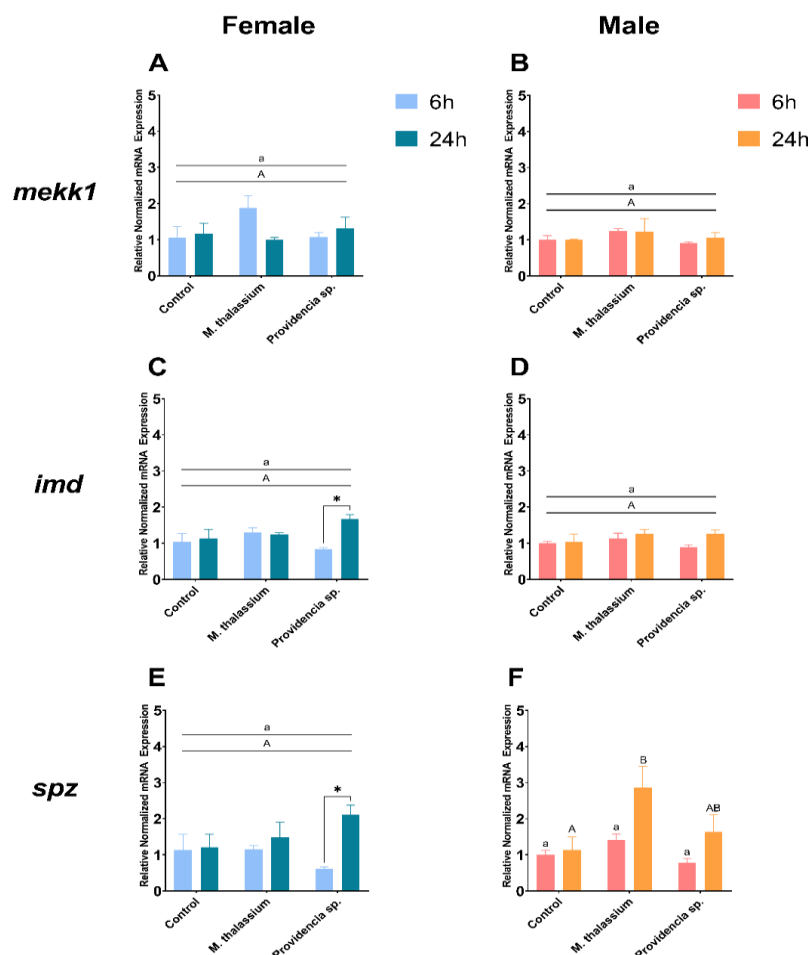


Figure III.4 - Expression of Imd and Toll pathways components. Relative normalized mRNA expression of *mekk1* (A, B), *imd* (C, D) and *spz* (E, F) in female (A, C, E) and male flies (B, D, F). Error bars represent SEM of means. Lowercase letters represent significant differences ($p < 0.05$) between conditions in 6 hours of exposure and uppercase letters represent significant differences ($p < 0.05$) between conditions in 24 hours of exposure and * represents significant differences ($p < 0.05$) between 6 and 24 hours on the same condition.

3.5. Multivariate Analysis Discloses Immune Response Associated with Bacterial Infection

A PCA was separately employed for each sex to disclose an overview of how infection with *M. thalassium* and *Providencia* sp. correlates with the expression of each target gene, TF and pathway component at each exposure time. In the PCA for females (Figure III.5A), PC1 describes 50.62% of the variance and PC2 27.00% of the variance.

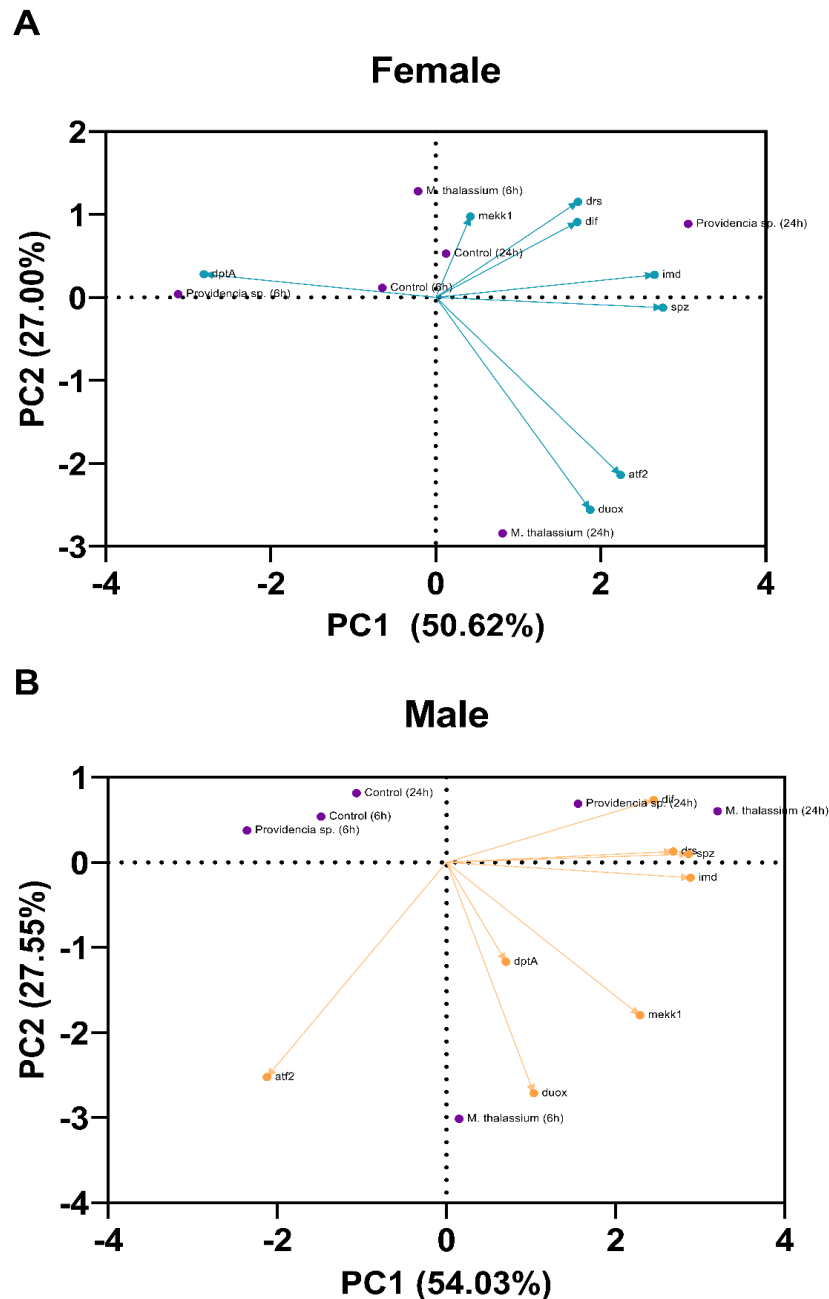


Figure III.5 - Principal Component Analysis of gene expression of Imd and Toll pathways components by oral infection for 6 and 24 hours. Biplots of females' (A) and males' (B) gene expression associate loadings of *duox*, *dptA*, *drs*, *atf2*, *dif*, *mekk1*, *imd* and *spz* and PC Scores of *M. thalassium* and *Providencia* sp. at 6 and 24 hours of exposure.

6 hours of infection with *M. thalassium*, although clustering near the Control groups, presents a correlation with expression of *mekk1*, while *Providencia* sp. strongly influenced *dptA* relative expression. On the other hand, 24 hours of exposure to *M. thalassium* clustered with the expression of *atf2* and *duox* in a single quadrant, while exposure to *Providencia* sp. clustered in the upper right quadrant with the Toll pathway components (*spz*, *dif* and *drs*) and *imd* (Figure III.5A). For males (Figure III.5B), PC1 illustrates 54.03% of the variance and PC2 27.55% of the variance. Contrarily to females, 6 hours of infection with *Providencia* sp. did not correlate with the expression of any gene and clustered with the Control groups. On the other hand, *M. thalassium* infection for 6 hours clustered with expression of *duox*, while *atf2*, *dptA* and *mekk1* did not cluster with any condition. 24 hours treatment with both bacteria was associated with the expression of the Toll pathway genes and *imd* (Figure III.5B).

4. Discussion

A demand for new strategies to control plant pests became imperative to design more sustainable and effective IPM programs and avoid the alarming application of pesticides on a variety of crops^{64–67}. Biological control is an innovative alternative, particularly the use of entomopathogenic microorganisms⁶⁸. Recent studies dwelled on the isolation of putative natural entomopathogenic bacteria to control the *D. suzukii* pest^{27–29}. In this study, *M. thalassium* and *Providencia* sp. were assessed for their potential as pathogens of *D. suzukii*. Moreover, immunity response of *D. suzukii* remains understudied and to characterize it, Imd and Toll pathways activity was assessed at the molecular level. Although well characterized for *D. melanogaster*, recent studies reveal different responses of the same pathways and AMPs expression between *D. suzukii* and *D. melanogaster*⁶⁹.

The obtained data showed a significant increase in CFU/fly levels in infection with both *M. thalassium* and *Providencia* sp. An interesting future analysis would be to further study the distinction between CFU count in the gut and hemolymph to distinguish a possible systemic infection. Nevertheless, no significant difference was found between 6 and 24 hours of exposure. These observations disclose comparable infection levels at both timepoints and sexes. Furthermore, in this study the infection was carried orally to mimic an on-field application of this bacteria as a biopesticide²⁹. Interestingly, neither of these bacteria caused a short-term (6 days) mortality in either female or male flies, contrarily to a previous survey of the mortality and AMP induction by *Providencia* spp. in *D. melanogaster*, which reported roughly 60% of mortality 6 days after systemic infection³³.

The main aim of this study was to evaluate the impact of infection and sex on the *D. suzukii* immune response, so RT-qPCR was employed to assess if these bacteria trigger immune molecular pathways and expression of AMPs. The expression of AMPs Diptericin (*dptA*) and Drosomycin (*drs*) and the Dual-oxidase (*duox*) are all involved in the regulation of the gut microbiota of *Drosophila* spp.^{46,53}. Dual-oxidase is involved in the production of ROS to the gut lumen to act as microbe-killing molecules^{70,71}. In this study, relative expression of *duox* was affected by the period of exposure in female flies, leading to increased levels only after 24 hours of exposure to both bacteria, even though infection reached plateau levels at 6 hours, revealing a delayed eliciting of the pathway. Furthermore, the upregulation of *duox* is congruent with delayed activation of its transcription factor *atf2* at 24 hours as well in females, suggesting that recognition of overpopulation of bacteria takes between 6 and 24 hours to elicit regulation. Conversely, AMPs were not differentially expressed in comparison to the uninfected control in female flies. In males, however, *duox* was differentially expressed depending on the bacteria; *M. thalassium* lead to an upregulation of *duox*, while *Providencia* sp. did not significantly alter *duox* expression, suggesting that *Providencia* sp. is able to overcome regulation by ROS. The upregulation of *atf2* in males at 6 hours of infection with *M. thalassium* shows, contrarily to females, early activation of the pathway. On the other hand, male flies also showed a bacteria and time-dependent upregulation of *spz* expression, once again revealing that *M. thalassium* is recognized by males' immune system, eliciting the Toll pathway. The upregulation of *spz* at 24 hours suggests that the Toll pathway is activated later than *duox* expression.

Although posthoc analysis failed to significantly identify AMP differential expression, PCA demonstrated a strong association of *Providencia* sp. 6 hours infection with the expression of *dptA* in females, disclosing an early AMP response to bacterial infection, while 6 hours of exposure to *M. thalassium* did not elicit any response. In contrast, 24 hours of exposure to *Providencia* sp. clustered with the Toll pathway components (*spz*, *dif* and *drs*), displaying a late activation of this pathway. Additionally, 24 hours of exposure to *M. thalassium* grouped with *atf2* and *duox*, which is a surprising observation, as *duox* prominently acts as the front line of the innate immune system, regulating the gut microbiota, with activation of Imd pathway for ROS-resistant microbes^{54,72}. These observations suggest a differential immune response to *Providencia* sp. in comparison to *M. thalassium*, disclosing an AMP-dependent immune response to *Providencia* sp., while a ROS-dependent regulation successfully regulates *M. thalassium* to undamaging levels. In contrast, PCA analysis of male expression revealed no immune response on infection by *Providencia* sp. at 6 hours, while earlier

infection with *M. thalassium* correlated with *duox* expression. Furthermore, similarly to females exposed 24 hours to *Providencia* sp., late infection of males for 24 hours with both bacteria strongly correlated with expression of the Toll pathway, revealing a late activation of this pathway upon infection, which is congruent with previous reports in *D. melanogaster*⁷³.

The previous analysis reveals a possible sex-dependent immune response to bacterial infection. Sex bias in the gene expression found here for *D. suzukii* is supported by the transcriptomic analysis of a recent study that showed a bias in the expression of certain genes, with AMPs being more expressed in females⁷⁴. However, the sex-dependent immune response has only been extensively studied and reviewed for *D. melanogaster*⁷⁵. Previous studies have reported an increased sensibility to bacterial infection by *imd* suppressed females, contrarily to male flies that present more susceptibility to bacterial infections when *spz* is suppressed⁷⁶. The genetic evolution of Toll receptors in male *D. melanogaster* may be one of the underlying reasons for this phenomenon⁷⁷. Additionally, infection of *D. melanogaster* with *P. rettgeri* is controlled by the Imd pathway in both sexes, while Toll pathway is upregulated in male flies, in comparison to females, impacting susceptibility to infection⁷⁸. Results of this study report a similar behaviour in *D. suzukii*, as female flies appear to elicit *dptA* (Imd pathway) as a response to *Providencia* sp., while males predominantly Toll pathway expression for both bacteria. Besides a divergence in the regulated immune pathways, the time of response also diverged between sexes. Infection in female flies elicited an earlier (less than 6 hours) AMP response than males (between 6 and 24 hours). This may be explained by an increased basal expression of the Imd pathway in females. This has been previously observed in *D. melanogaster* and is possibly due to their greater and specialized reproductive involvement, conferring increased resistance to microbial infections^{79,80}. Finally, the susceptibility and immune response elicited by both bacteria are sex-dependent as results suggest a ROS-regulation of *M. thalassium* in females only at 24 hours of infection, eliciting no AMP expression, while males reveal more sensitivity to this bacterium as they start an early ROS-dependent regulation, shifting to AMP (*drs*) expression in late infection.

In summary, the results from this report reveal that after six days, the flies infected with the *M. thalassium* or *Providencia* bacterial strains, despite suffering not mortality, immune molecular responses were elicited. Other indicators, such as, fecundity, number of offspring, development success and development time would further disclose if infection with these bacteria disclosed any deleterious effect. Nevertheless,

important information was obtained from the transcript expression analysis about the immune dynamics of *D. suzukii*. Namely, about AMPs and Dual-oxidase expression, which are important gut-microbe regulators to combat infection and modulate microbial disbalance. Similarly to *D. Melanogaster*, sex-dependent immunity is also a characteristic of *D. suzukii*, described by an early increased eliciting of the Imd pathway only in females and a strong late eliciting of the Toll pathway predominantly in male flies. Future work with Imd and Toll mutant flies would provide validation of these results. Furthermore, sex-dependency was also displayed as males revealed higher susceptibility to *M. thalassium* than female flies. A sex-dependent immune response is utterly important when developing biocontrol strategies to find more efficient entomopathogenic agents that overcome resistance mechanisms. Although this study was limited to the evaluation of Imd and Toll pathways, stress and immune molecular responses comprising a more complex and sophisticated network of pathways and interactions are of great interest to thoroughly characterize the response of *D. suzukii* to natural pathogens and understand how to overcome resistance mechanisms, including the expression of other pathways.

References

1. Santos, L. A. dos *et al.* Global potential distribution of *Drosophila suzukii* (Diptera, Drosophilidae). *PLoS One* **12**, 1–13 (2017).
2. Asplen, M. K. *et al.* Invasion biology of spotted wing *Drosophila* (*Drosophila suzukii*): a global perspective and future priorities. *J Pest Sci (2004)* **88**, 469–494 (2015).
3. de Ros, G., Grassi, A. & Pantezzi, T. Recent Trends in the Economic Impact of *Drosophila suzukii*. *Drosophila suzukii Management* 11–27 (2020).
4. Bolda, M. P., Goodhue, R. E. & Zalom, F. G. Spotted Wing *Drosophila*: Potential Economic Impact of a Newly Established Pest. *ARE Update* **13**, 5–8 (2010).
5. de Ros, G., Anfora, G., Grassi, A. & Ioriatti, C. The potential economic impact of *Drosophila suzukii* on small fruits production in Trentino (Italy). *Integrated protection of fruit crops IOBC-WPRS Bulletin* **91**, 317–321 (2013).
6. Yeh, D. A., Drummond, F. A., Gómez, M. I. & Fan, X. The Economic Impacts and Management of Spotted Wing *Drosophila* (*Drosophila suzukii*): The Case of Wild Blueberries in Maine. *J Econ Entomol* **113**, 1262–1269 (2020).
7. Mazzi, D., Bravin, E., Meraner, M., Finger, R. & Kuske, S. Economic Impact of the Introduction and Establishment of *Drosophila suzukii* on Sweet Cherry Production in Switzerland. *Insects* **2017** **8**, 18 (2017).
8. de Ros, G., Conci, S., Pantezzi, T. & Savini, G. The economic impact of invasive pest *Drosophila suzukii* on berry production in the Province of Trento, Italy. *J Berry Res* **5**, 89–96 (2015).
9. Knapp, L., Mazzi, D. & Finger, R. The economic impact of *Drosophila suzukii*: perceived costs and revenue losses of Swiss cherry, plum and grape growers. *Pest Manag Sci* **77**, 978 (2021).
10. Farnsworth, D. *et al.* Economic analysis of revenue losses and control costs associated with the spotted wing *Drosophila*, *Drosophila suzukii* (Matsumura), in the California raspberry industry. *Pest Manag Sci* **73**, 1083–1090 (2017).
11. Nowak, J. T., McCravy, K. W., Fettig, C. J. & Berisford, C. W. Susceptibility of Adult Hymenopteran Parasitoids of the Nantucket Pine Tip Moth (Lepidoptera: Tortricidae) to Broad-Spectrum and Biorational Insecticides in a Laboratory Study. *J. Econ. Entomol* **94**, 1122–1129 (2001).

12. Disi, J. O. & Sial, A. A. Laboratory Selection and Assessment of Resistance Risk in *Drosophila suzukii* (Diptera: Drosophilidae) to Spinosad and Malathion. *Insects* **12**, 794 (2021).
13. Haviland, D. R. & Beers, E. H. Chemical Control Programs for *Drosophila suzukii* that Comply With International Limitations on Pesticide Residues for Exported Sweet Cherries. *J Integr Pest Manag* **3**, 1–6 (2012).
14. Gress, B. E. & Zalom, F. G. Identification and risk assessment of spinosad resistance in a California population of *Drosophila suzukii*. *Pest Manag Sci* **75**, 1270–1276 (2019).
15. Knapp, L., Mazzi, D. & Finger, R. Management strategies against *Drosophila suzukii*: insights into Swiss grape growers choices. *Pest Manag Sci* **75**, 2820–2829 (2019).
16. Diepenbrock, L. M., Rosensteel, D. O., Hardin, J. A., Sial, A. A. & Burrack, H. J. Season-long programs for control of *Drosophila suzukii* in southeastern U.S. blueberries. *Crop Protection* **81**, 76–84 (2016).
17. Tait, G. *et al.* *Drosophila suzukii* (Diptera: Drosophilidae): A Decade of Research Towards a Sustainable Integrated Pest Management Program. *J Econ Entomol* **114**, 1950–1974 (2021).
18. Moore, S. D. Biological Control of a Phytosanitary Pest (*Thaumatotibia leucotreta*): A Case Study. *Int J Environ Res Public Health* **18**, 1198 (2021).
19. Abd-Elgawad, M. M. M. Towards optimization of entomopathogenic nematodes for more service in the biological control of insect pests. *Egypt J Biol Pest Control* **29**, 1–8 (2019).
20. Rodríguez-González, A. *et al.* Investigations of *Trichoderma* spp. and *Beauveria bassiana* as biological control agent for *Xylotrechus arvicola*, a major insect pest in Spanish vineyards. *J Econ Entomol* **111**, 2585–2591 (2018).
21. Santos, A. C. da S., Diniz, A. G., Tiago, P. V. & Oliveira, N. T. de. Entomopathogenic *Fusarium* species: a review of their potential for the biological control of insects, implications and prospects. *Fungal Biol Rev* **34**, 41–57 (2020).
22. Valtierra-De-luis, D., Villanueva, M., Berry, C. & Caballero, P. Potential for *Bacillus thuringiensis* and Other Bacterial Toxins as Biological Control Agents to Combat Dipteran Pests of Medical and Agronomic Importance. *Toxins (Basel)* **12**, 773 (2020).

23. Malaikozhundan, B. & Vinodhini, J. Biological control of the Pulse beetle, *Callosobruchus maculatus* in stored grains using the entomopathogenic bacteria, *Bacillus thuringiensis*. *Microb Pathog* **114**, 139–146 (2018).
24. Rotich, E., Mmbaga, M. T. & Joshua, J. Biological control of powdery mildew on *Cornus florida* using endophytic *Bacillus thuringiensis*. *Canadian Journal of Plant Pathology* **42**, 182–191 (2019).
25. Jakka, S. R. K., Shrestha, R. B. & Gassmann, A. J. Broad-spectrum resistance to *Bacillus thuringiensis* toxins by western corn rootworm (*Diabrotica virgifera virgifera*). *Sci Rep* **6**, 1–9 (2016).
26. Lacey, L. A., Frutos, R., Kaya, H. K. & Vail, P. Insect Pathogens as Biological Control Agents: Do They Have a Future? *Biological Control* **21**, 230–248 (2001).
27. Hiebert, N., Carrau, T., Bartling, M., Vilcinskas, A. & Lee, K. Z. Identification of entomopathogenic bacteria associated with the invasive pest *Drosophila suzukii* in infested areas of Germany. *J Invertebr Pathol* **173**, 1–11 (2020).
28. Hiebert, N. *et al.* The Gram-Positive Bacterium *Leuconostoc pseudomesenteroides* Shows Insecticidal Activity against Drosophilid and Aphid Pests. *Insects* **11**, 1–15 (2020).
29. Bing, X. L., Winkler, J., Gerlach, J., Loeb, G. & Buchon, N. Identification of natural pathogens from wild *Drosophila suzukii*. *Pest Manag Sci* **77**, 1594–1606 (2021).
30. Huang, C. Extensively drug-resistant *Alcaligenes faecalis* infection. *BMC Infect Dis* **20**, 1–11 (2020).
31. Li, G. *et al.* Complete genome sequence of *Achromobacter spanius* type strain DSM 23806T, a pathogen isolated from human blood. *J Glob Antimicrob Resist* **14**, 1–3 (2018).
32. Wang, Y. *et al.* Probiotic Potential of *Leuconostoc pseudomesenteroides* and *Lactobacillus* Strains Isolated From Yaks. *Front Microbiol* **9**, 2987 (2018).
33. Galac, M. R. & Lazzaro, B. P. Comparative pathology of bacteria in the genus *Providencia* to a natural host, *Drosophila melanogaster*. *Microbes Infect* **13**, 673–683 (2011).
34. Somvanshi, V. S. *et al.* *Providencia vermicola* sp. nov., isolated from infective juveniles of the entomopathogenic nematode *Steinernema thermophilum*. *Int J Syst Evol Microbiol* **56**, 629–633 (2006).

35. Andolfo, G., Schuster, C., Gharsa, H. ben, Ruocco, M. & Leclerque, A. Genomic analysis of the nomenclatural type strain of the nematode-associated entomopathogenic bacterium *Providencia vermicola*. *BMC Genomics* **22**, 1–21 (2021).
36. Secil, E. S., Sevim, A., Demirbag, Z. & Demir, I. Isolation, characterization and virulence of bacteria from *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Biologia (Bratisl)* **67**, 767–776 (2012).
37. Klüber, P., Müller, S., Schmidt, J., Zorn, H. & Rühl, M. Isolation of Bacterial and Fungal Microbiota Associated with *Hermetia illucens* Larvae Reveals Novel Insights into Entomopathogenicity. *Microorganisms* **10**, 1–19 (2022).
38. Tamez-Guerra, P. *et al.* Detection of genes encoding antimicrobial peptides in Mexican strains of *Trichoplusia ni* (Hübner) exposed to *Bacillus thuringiensis*. *J Invertebr Pathol* **98**, 218–227 (2008).
39. Ericsson, J. D., Janmaat, A. F., Lowenberger, C. & Myers, J. H. Is decreased generalized immunity a cost of Bt resistance in cabbage loopers *Trichoplusia ni*? *J Invertebr Pathol* **100**, 61–67 (2009).
40. Gliński, Z. & Buczek, K. Response of the Apoidea to fungal infections. *Apiacta* **38**, 183–189 (2003).
41. Morin-Poulard, I., Vincent, A. & Crozatier, M. The *Drosophila* JAK-STAT pathway in blood cell formation and immunity . *JAKSTAT* **2**, e25700 (2013).
42. Garriga, A., Mastore, M., Morton, A., del Pino, F. G. & Brivio, M. F. Immune Response of *Drosophila suzukii* Larvae to Infection with the Nematobacterial Complex *Steinernema carpocapsae*–*Xenorhabdus nematophila*. *Insects* **11**, 210 (2020).
43. Capo, F., Wilson, A. & di Cara, F. The Intestine of *Drosophila melanogaster*: An Emerging Versatile Model System to Study Intestinal Epithelial Homeostasis and Host-Microbial Interactions in Humans. *Microorganisms* **7**, 336 (2019).
44. Hultmark, D. *Drosophila* immunity: paths and patterns. *Curr Opin Immunol* **15**, 12–19 (2003).
45. Lin, S. J. H., Cohen, L. B. & Wasserman, S. A. Effector specificity and function in *Drosophila* innate immunity: Getting AMPed and dropping Bombs. *PLoS Pathog* **16**, e1008480 (2020).
46. Tzou, P. *et al.* Tissue-Specific Inducible Expression of Antimicrobial Peptide Genes in *Drosophila* Surface Epithelia. *Immunity* **13**, 737–748 (2000).

47. Choe, K. M., Werner, T., Stöven, S., Hultmark, D. & Anderson, K. v. Requirement for a peptidoglycan recognition protein (PGRP) in relish activation and antibacterial immune responses in *Drosophila*. *Science* (1979) **296**, 359–362 (2002).
48. Gottar, M. *et al.* The *Drosophila* immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. *Nature* **416**, 640–644 (2002).
49. Bosco-Drayon, V. *et al.* Peptidoglycan sensing by the receptor PGRP-LE in the *Drosophila* gut induces immune responses to infectious bacteria and tolerance to microbiota. *Cell Host Microbe* **12**, 153–165 (2012).
50. Kleino, A. & Silverman, N. The *Drosophila* IMD pathway in the activation of the humoral immune response. *Dev Comp Immunol* **42**, 25–35 (2014).
51. Lhocine, N. *et al.* PIMS Modulates Immune Tolerance by Negatively Regulating *Drosophila* Innate Immune Signaling. *Cell Host Microbe* **4**, 147–158 (2008).
52. Ertü Rk-Hasdemir, D. *et al.* Two roles for the *Drosophila* IKK complex in the activation of Relish and the induction of antimicrobial peptide genes. *Proc Natl Acad Sci U S A* **106**, 9779–9784 (2009).
53. Kim, S. H. & Lee, W. J. Role of DUOX in gut inflammation: Lessons from *Drosophila* model of gut-microbiota interactions. *Front Cell Infect Microbiol* **3**, 116 (2014).
54. Ha, E. M. *et al.* Coordination of multiple dual oxidase–regulatory pathways in responses to commensal and infectious microbes in *Drosophila* gut. *Nat Immunol* **10**, 949–957 (2009).
55. Han, J., Lee, J. D., Bibbs, L. & Ulevitch, R. J. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265**, 808–811 (1994).
56. Chen, J. *et al.* Participation of the p38 pathway in *Drosophila* host defense against pathogenic bacteria and fungi. *Proc Natl Acad Sci U S A* **107**, 20774–20779 (2010).
57. Han, Z. S. *et al.* A Conserved p38 Mitogen-Activated Protein Kinase Pathway Regulates *Drosophila* Immunity Gene Expression. *Mol Cell Biol* **18**, 3527 (1998).
58. Filipe, S. R., Tomasz, A. & Ligoxygakis, P. Requirements of peptidoglycan structure that allow detection by the *Drosophila* Toll pathway. *EMBO Rep* **6**, 327–333 (2005).
59. Valanne, S., Wang, J.-H. & Rämet, M. The *Drosophila* Toll Signaling Pathway . *The Journal of Immunology* **186**, 649–656 (2011).

60. Jang, I. H. *et al.* A Spätzle-processing enzyme required for toll signaling activation in *Drosophila* innate immunity. *Dev Cell* **10**, 45–55 (2006).
61. Ip, Y. T. *et al.* Dif, a dorsal-related gene that mediates an immune response in *Drosophila*. *Cell* **75**, 753–763 (1993).
62. Untergasser, A. *et al.* Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res* **35**, (2007).
63. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45 (2001).
64. Sun, S., Zhang, C. & Hu, R. Determinants and overuse of pesticides in grain production: A comparison of rice, maize and wheat in China. *China Agricultural Economic Review* **12**, 367–379 (2020).
65. Schreinemachers, P. *et al.* How much is too much? Quantifying pesticide overuse in vegetable production in Southeast Asia. *J Clean Prod* **244**, 118738 (2020).
66. Sookhtanlou, M., Allahyari, M. S. & Surujlal, J. Health Risk of Potato Farmers Exposed to Overuse of Chemical Pesticides in Iran. *Saf Health Work* **13**, 23–31 (2022).
67. Pimentel, D. Environmental and Economic Costs of the Application of Pesticides Primarily in the United States. *Environ Dev Sustain* **7**, 229–252 (2005).
68. van Lenteren, J. C., Bolckmans, K., Köhl, J., Ravensberg, W. J. & Urbaneja, A. Biological control using invertebrates and microorganisms: plenty of new opportunities. *BioControl* **63**, 39–59 (2018).
69. Kim, Y., Goh, G. & Kim, Y. H. Expression of antimicrobial peptides associated with different susceptibilities to environmental chemicals in *Drosophila suzukii* and *Drosophila melanogaster*. *Pestic Biochem Physiol* **187**, 105210 (2022).
70. Leto, T. L. & Geiszt, M. Role of Nox Family NADPH Oxidases in Host Defense. *Antioxid Redox Signal* **8**, 1549–1561 (2006).
71. Geiszt, M., Witta, J., Baffi, J., Lekstrom, K. & Leto, T. L. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *The FASEB Journal* **17**, 1–14 (2003).
72. Ryu, J. H. *et al.* An essential complementary role of NF-κB pathway to microbicidal oxidants in *Drosophila* gut immunity. *EMBO J* **25**, 3693–3701 (2006).

73. de Gregorio, E., Spellman, P. T., Tzou, P., Rubin, G. M. & Lemaitre, B. The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *EMBO J* **21**, 2568–2579 (2002).
74. Deng, D. *et al.* Transcriptome analysis of sex-biased gene expression in the spotted-wing *Drosophila*, *Drosophila suzukii* (Matsumura). *G3 Genes|Genomes|Genetics* **12**, (2022).
75. Belmonte, R. L., Corbally, M. K., Duneau, D. F. & Regan, J. C. Sexual Dimorphisms in Innate Immunity and Responses to Infection in *Drosophila melanogaster*. *Front Immunol* **10**, 3075 (2020).
76. Taylor, K. & Kimbrell, D. Host Immune Response and Differential Survival of the Sexes in *Drosophila*. *Fly (Austin)* **1**, 197–204 (2007).
77. Levin, T. C. & Malik, H. S. Rapidly Evolving Toll-3/4 Genes Encode Male-Specific Toll-Like Receptors in *Drosophila*. *Mol Biol Evol* **34**, 2307–2323 (2017).
78. Duneau, D. F. *et al.* The Toll pathway underlies host sexual dimorphism in resistance to both Gram-negative and Gram-positive bacteria in mated *Drosophila*. *BMC Biol* **15**, 1–17 (2017).
79. Vincent, C. M. & Sharp, N. P. Sexual antagonism for resistance and tolerance to infection in *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences* **281**, (2014).
80. Vincent, C. M. & Dionne, M. S. Disparate regulation of IMD signaling drives sex differences in infection pathology in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **118**, e2026554118 (2021).

IV. Chapter 4: Assessing the proliferation capacity of putative stable gut bacteria of *Drosophila suzukii*

Abstract

New Biological control strategies are currently in the scope of research, towards providing sustainable and effective alternatives to agrochemicals in the control of the *Drosophila suzukii* pest. Manipulation of the microbiome poses an interesting strategy to overcome insecticide resistance, ecological damages, and public health problems. Disrupting the mutualistic microbe populations needed for the host's homeostasis is an innovative approach. In this study, we established colonies of monoassociated *D. suzukii* flies with symbiont candidates, and monitored their gut colonizing capacity. This proliferation assay relies on the quantification of bacterial levels after oral ingestion of a specific bacteria. To do this, we adapted a method from another *Drosophila* model to test putative stable bacteria by characterizing its gut colonizing capacity. *Leuconostoc pseudomesenteroides* showed proliferation ability in female flies, conversely to male flies, suggesting a sex-specific interaction with bacteria. Moreover, the proliferation behaviour of two *Acetobacter thailandicus* isolated from *D. suzukii* sampled from different regions was significantly different, suggesting a strain-specific interaction with the host. This study represents an important step for further studies toward identifying new symbionts of this pest that can be used as targets for pest control.

1. Introduction

Soft-skinned fruits and berries producers are currently threatened by the insect pest *Drosophila suzukii*¹. Since its first characterization at the beginning of the 20th century in East Asia^{2,3}, it has then disseminated to other continents⁴, causing severe damage to several cultures, with particular incidence on red fruits, and leading to high economic losses^{5–11}. Current pest control strategies still rely mainly on the application of chemical pesticides, which comprise a plethora of drawbacks at the ecological level, as most of these formulations have a broad-spectrum range, targeting not only the pest but also other beneficial and native species such as natural predators or pollinizers^{12,13}. Furthermore, there is also the concern of insecticide resistance, which tends to increase with continued and excessive application¹⁴, and concerns at the public health level regarding the risks posed by these agrochemicals to consumers due for example to their residual levels on fruits¹⁵. Alternative management strategies are,

consequently, of urgent and vital importance to ensure effective and safe pest control, as part of Integrated Pest Management (IPM) programs^{16–18}. A current line of research on sustainable pest management is biological control tools^{19,20}, particularly by integrating the use of entomopathogenic microorganisms as a form of controlling pests^{21–23}. For *D. suzukii*, general entomopathogens, such as *Bacillus thuringiensis*, a commercially available entomopathogen, and *Photorhabdus luminescens*, an entomopathogenic nematode symbiont, have already presented insecticidal activity^{22,24,25}.

As stated above, most of the research on the use of microorganisms to control insect pests focuses on pathogenic microorganisms. However, mutualistic microorganisms should not be discarded as they are involved in different processes of resistance and adaptation, both important factors when studying insect pests^{27–31}. Therefore, targeting mutualistic microorganisms can efficiently disrupt the gut microbiota and functional metabolism of the insect, or manipulate desired advantageous traits, reducing the pest's fitness in the environment³².

Identifying symbiotic and mutualistic microbes reveals to be a challenging demand, as the identification of microbes present in the gut does not provide, for itself, enough data to confirm the establishment of symbiotic links between species and host. This is due to the variability of microbial communities in insects' gut, which distinguishes transient microbiota, that does not stably associate with the host, from colonizing microbiota, that stably persists in the host^{33,34}.

Drosophila melanogaster is the most well-explored *Drosophila* spp., having a robustly studied and simple microbiota that is used for translational studies. Therefore, its highly optimized manipulation and reproducible assays, enabling the study of immunity, nutritional and pathogenic interactions with its microbiota^{35,36}. Moreover, the identification of symbiotic links is facilitated using thoroughly studied models, overcoming barriers such as the influence of the whole network of microbial species interacting with each other and the host³⁷. Thus, generating axenic (or germ-free) and gnotobiotic (with a known composition of the microbiome, e.g., mono-associations) shifted the landscape to study host-microbe interactions. Harnessing the gnotobiotic flies advantages eliminates side factors, such as the influence of the bacteria community in the gut of flies, enables the study of specific interactions with certain strains of interest, as well as unveils the role and effects of symbiotic bacteria^{38,39}. Besides, monoassociation of *D. suzukii* with microbes enabled the study of specific interactions, such as the effect of a single species in different nutrient environments⁴⁰.

or to reveal specific associations of *D. suzukii* microbiota in *D. melanogaster*⁴¹. To unveil the symbiotic relationship of single species of bacteria with *D. melanogaster*, Pais *et al.* (2018) proved the establishment of specific mutualistic links between two *Acetobacter* species (*Acetobacter thailandicus* and *Acetobacter cibinongensis*) and *D. melanogaster*, proving them to constitute part of the colonizing and stable microbiota of the fly's gut⁴².

Applying these concepts and methodologies to study symbiotic microorganisms of *D. suzukii* is of great importance for the expansion of possible microbial targets for pest control. Even though *Acetobacteraceae*, *Enterobacteriaceae* and *Lactobacillaceae* present, overall, as the dominant families in *D. suzukii*'s gut, the currently available data on microbiota composition of this species is still variable^{40,43–47}. The dominant taxa of bacteria varies across the different samples and location. For instance, Chandler *et al* (2014) and Hiebert *et al* (2020) revealed dominance of the *Tatumella* genus^{43,47}, while Bing *et al* (2018) disclosed dominance of the *Dickeya* genus⁴⁰ and Martinez-Sañudo *et al* (2018) disclosed dominance of *Acetobacter* and *Gluconobacter*⁴⁶. This variability poses as an obstacle for the identification of mutualistic species. It is proposed here that one of the main reasons for the lack of an established core microbiome is also the absence of optimized protocols for these kinds of studies for *D. suzukii*. In fact, to date, only two published studies have generated axenic flies^{40,48}, but no studies have used gnotobiotic or monoassociated flies to assess the colonizing ability of bacteria in the gut of this species.

To fill this gap, the aim of this study was to optimize for *D. suzukii* a protocol to generate axenic *D. suzukii* flies and test putative stable bacteria for their colonizing capacity. This proliferation assay relies on the quantification of bacteria after oral ingestion of a specific bacterium species to understand if it is able to colonize the host's gut, in this case, *D. suzukii*. This is a pilot study to unveil new mutualistic relationships established by this pest, opening new doors to further develop new targets for sustainable pest management.

2. Materials and Methods

2.1. *D. suzukii* Collection and Rearing

A fertilized *D. suzukii* female was captured in Oeiras, Portugal (38°41'51.1"N 9°18'55.4"W), in September 2021 and another female was captured in Castelo de Paiva, Portugal in October 2021 (41°03'14.3"N 8°16'49.2"W), both using traps with mashed fruit to attract flies and an exclusion net to prevent contact with bacteria. To

establish a laboratory colony, the captured fertilized female was transferred to an artificial fly medium (6.8% sucrose, 6.4% cornmeal, 1.8% yeast, 4% molasses, and 0.9% agar in distilled water), which otherwise stated was used throughout the study. The offspring were crossed to generate an isofemale line and stocks were kept at 22°C with uncontrolled humidity and natural light cycle.

2.2. Sample Processing for Bacterial Isolation

To isolate the stable bacteria of wild flies, the captured female (Oeiras, 2021) (from which the isofemale line was generated) was transferred daily to new bottles with fly medium to clear the fly of the non-stable microbiota. After 10 days, the fly was collected and surface sterilized with absolute ethanol to eliminate surface bacteria and processed by homogenization using a TissueLyser II (Qiagen, Hilden, Germany) in microtubes with 250 µL of Lysogeny Broth (LB) serially diluted and Tungsten Carbide Beads (Qiagen, Hilden, Germany) at a 1200 oscillations/min for 1 min. The homogenate was plated in two different media: de Man, Rogosa and Sharpe (MRS) (Merck, Darmstadt, Germany) (1.5% agar) and Mannitol (5% D-Mannitol, 0.3% peptone, 0.5% yeast extract and 1.5% agar). For each individual morphotype, a colony was collected for bacterial identification. Bacteria from the captured female (Castelo de Paiva, 2021) were isolated immediately after collection and surface sterilization with 100% EtOH and homogenization with a pestle in 150 µL of sterile phosphate buffer saline (PBS). The homogenate was then plated in LB, Brain Heart Infusion (BHI) and MRS plates.

2.3. Identification of Putative Stable Bacteria

To obtain genomic DNA, fresh colonies from both media (MRS and Mannitol) were transferred to a 1:1 Cell Lysis Solution (Promega) and Proteinase K for 50 min at 65°C for lysis, followed by inactivation of the enzymes for 10 minutes at 95°C. DNA samples were then amplified for the partial 16S ribosomal RNA (rRNA) gene with universal primers 27F (5' - GAGAGTTTGATCCTGGCTCAG - 3') and 1495R (5' - CTACGGCTACCTTGTTACGA - 3'), using 1X Taq Master Mix (Bioron, Römerberg, Germany), at 4 mM MgCl₂ and 400 µM of each dNTP, resorting to the following thermal cycling program: 95°C for 5 min; 30 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 2 min; 72°C for 10 min. PCR products were then purified with an ExoSap solution (0.1 µL of 20 U/µL Exonuclease I (NEB) and 0.1 µL of 1 U/µL rAPid Alkaline Phosphatase (Roche) in 35 µL of milliQ water). ExoSap solution was added to the PCR products in a 1:4 ratio and incubated at 37°C for 30 minutes followed by inactivation at 95°C for 5

minutes. Purified DNA samples were then outsourced for Sanger Sequencing at the GATC, Eurofins Genomics (Ebersberg, Germany).

The isolation and identification of bacteria (Sections 2.2 and 2.3) from Oeiras, Portugal 2021 *D. suzukii* was performed by Rita Valente at IGC. The *A. thailandicus* isolated from Castelo de Paiva, Portugal 2021 was isolated according to Chapter I (Sections 2.2 and 2.3).

2.4. Establishment of a Protocol to Generate Axenic *D. suzukii*

Axenic *D. suzukii* flies were generated according to the protocol of Pais *et al.* (2018) to develop *D. melanogaster* axenic flies⁴². To collect *D. suzukii* eggs, adults were reared in set up cages with apple juice agar plates (3% agar) 4 days prior to egg collection, plates were changed daily. On the egg collection day, cages and plates were both changed and incubated for 3 to 5 hours at 22°C for posterior egg collection under the scope. The eggs were individually collected under the stereo microscope to a cell strainer and were dechorionated and sterilized using a 2% bleach solution with constant mixing until the chorion was no longer visible. The dechorionated eggs were then washed with milliQ water and transferred to a 70% EtOH for 5 min. After sterilization, the eggs were retransferred to a sterile cell strainer and thoroughly washed with sterile milliQ water before transferring to sterile fly food and incubated at 22°C. Axenic flies were collected 4 days after the first emerging adults. The experimental procedure is detailed in Figure IV.1.

2.5. Establishment of gnotobiotic associations and Proliferation Assay

Two putative stable bacteria isolated from the captured female from Oeiras, 2021 (*A. thailandicus* and *Leuconostoc pseudomesenteroides*) and one from the captured female Castelo de Paiva 2021 (*A. thailandicus*, henceforward referred as *A. thailandicus* (CP21)) were selected based on previous results in *D. melanogaster*⁴². The tested bacteria were grown overnight in Mannitol (*Acetobacter* sp.) or MRS (*Leuconostoc* sp.) broth media at 30°C at 180 rpm.

2.5.1. Feeding Conditions

Three to six days old axenic *D. suzukii* flies were transferred to a new vial with sterile fly food covered with a filter paper embedded with a solution of each individual bacterium at an optical density at 600 nm (OD₆₀₀) of 0.01 and 2.5% sucrose. To account for an unknown feeding behaviour of *D. suzukii* and to obtain consistent and reproducible levels of ingested bacteria, feeding time was first tested for three different conditions, using *A. thailandicus*: *i*) 4 hours of feeding, *ii*) 6 hours of feeding, and *iii*)

overnight starved in a vial with a sterile filter paper embedded in tap water before transfer to bacterial solution for 4 hours of feeding.

2.5.2. Proliferation Time

After determining the overnight starvation prior to 4 hours feeding as optimal feeding conditions, 6 to 8 fed flies were then placed into single bottles with sterile fly food for 16 and 20 hours at 22°C alongside with chaser flies (axenic flies marked with a red dot in both wings and thorax, with no previous contact with bacteria), to ensure that proliferation is not due to bacterial growth on the fly food and reingestion cycle. The remaining flies were processed to evaluate the bacterial levels immediately after feeding and before proliferation (0 hours). To assess the bacterial levels in the flies at 0, 16 and 20 hours, the flies were surface sterilized with ethanol and macerated as previously described in section 2.2., individually plated in the Mannitol (*Acetobacter* spp.) or MRS (*Leuconostoc* spp.) and incubated at 25°C (Figure IV.1).

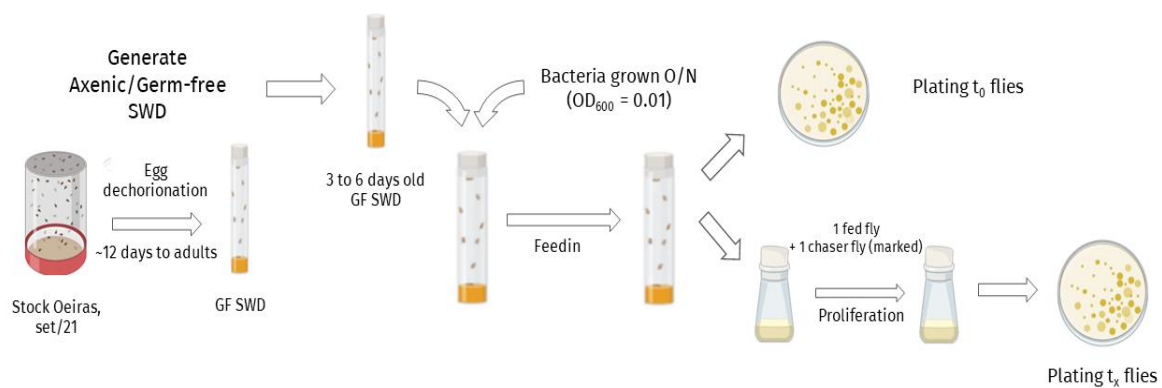


Figure IV.1 – Axenic flies and proliferation assay experimental design.

2.6. Statistical Analysis

Statistical analysis was performed using R software⁴⁹. For the testing of the different feeding conditions, one sample t-test was performed individually for each condition and sex to assess if the mean is greater than the minimal detectable level. Bacterial levels were analysed using a linear mixed-model (lmm) the *lme4* package⁵⁰, with time as fixed effect and replicate as a random effect. When analysing the sex-dependent proliferation behaviour, lmm was run with sex as the fixed effect and replicate as a random effect. For single replicate experiments, a linear model (lm) was used. Post-hoc pairwise comparisons were performed using the *emmeans* package⁵¹ with adjustment with Tukey. All plots were generated using the *ggplot2* package⁵².

3. Results

3.1. Optimal Feeding Conditions

The results of colony forming units (CFU) per fly (CFU/fly) in the different feeding conditions is depicted in Figure IV.2. Feeding for 4 hours was inefficient, as CFU/fly are not statistically significant different ($p > 0.05$) from the minimal detectable threshold, in neither female (0.728 log CFU/fly) or male flies (0.708 log CFU/fly). After feeding for 6 hours, male flies showed consistent bacterial levels (2.104 log CFU/fly, $p < 0.0001$), contrarily to females, whose values were similar to flies who were allowed to feed on the bacteria for 4 hours (0.747 log CFU/fly). Overnight starvation followed by 4 hours of feeding revealed the most stable results ($p < 0.05$) on both female (1.599 log CFU/fly) and male (1.662 log CFU/fly) flies (Figure IV.2).

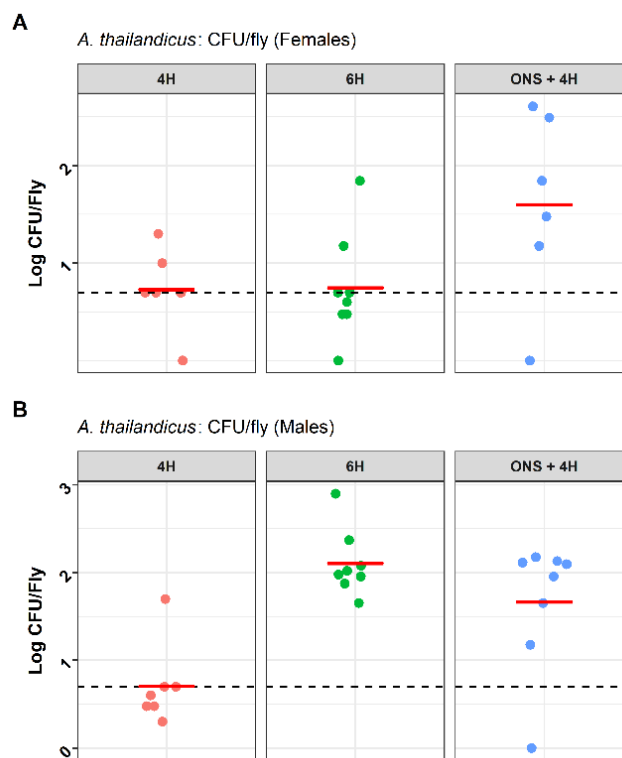


Figure IV.2 - Quantification of CFU/fly according to different feeding conditions. Quantification of CFU/fly according to different feeding conditions. Tested 6 hours feeding (6H), 4 hours feeding (4H) and overnight starvation + 4 hours feeding (ONS + 4H) in females (A) and males (B). Dots and red bar represent a single fly and the mean, respectively. Line shows the minimal detection limit of log CFUs = $\log_{10} 5$.

3.2. Influence of the Reingestion Cycle on the Proliferation

To assess the optimal proliferation time and the influence of reingested bacteria from the fly medium, 20 hours of proliferation was firstly tested with *A. thailandicus* and *L.*

pseudomesenteroides resorting to chaser flies to track the recontamination effect. Results for the proliferation of *A. thailandicus* did not reveal any significant change in bacterial levels from 0 to 20 hours ($\text{Im}, p > 0.05$) in neither female (1.56 log CFU/fly and 1.64 log CFU/fly) (Figure IV.3A) or male flies (1.75 log CFU/fly and 1.30 log CFU/fly) (Figure IV.3C), alongside with their respective chasers (1.27 log CFU/fly and 1.57 log CFU/fly). Conversely, *L. pseudomesenteroides* did show a statistically significant ($\text{Im}, p < 0.01$) increase in levels between 0 hours and 20 hours in female flies (2.61 log CFU/fly and 4.76 log CFU/fly) (Figure IV.3B), while males did not significantly ($\text{Im}, p > 0.05$) present altered levels (2.32 log CFU/fly and 1.43 log CFU/fly) (Figure IV.3D). Despite showing increased levels at 20 hours of proliferation in female flies fed with *L. pseudomesenteroides*, chasers' bacterial levels, although close ($\text{Im}, p = 0.051$), did not significantly show a difference from the 20 hours bacterial levels (3.24 log CFU/fly and 4.76 log CFU/fly, respectively), failing to rule out the possibility of reingestion of bacteria. Male flies fed with *L. pseudomesenteroides* did not show a significant difference between chasers' and 20 hours bacterial levels ($\text{Im}, p > 0.05$; 1.69 log CFU/fly and 1.43 log CFU/fly, respectively).

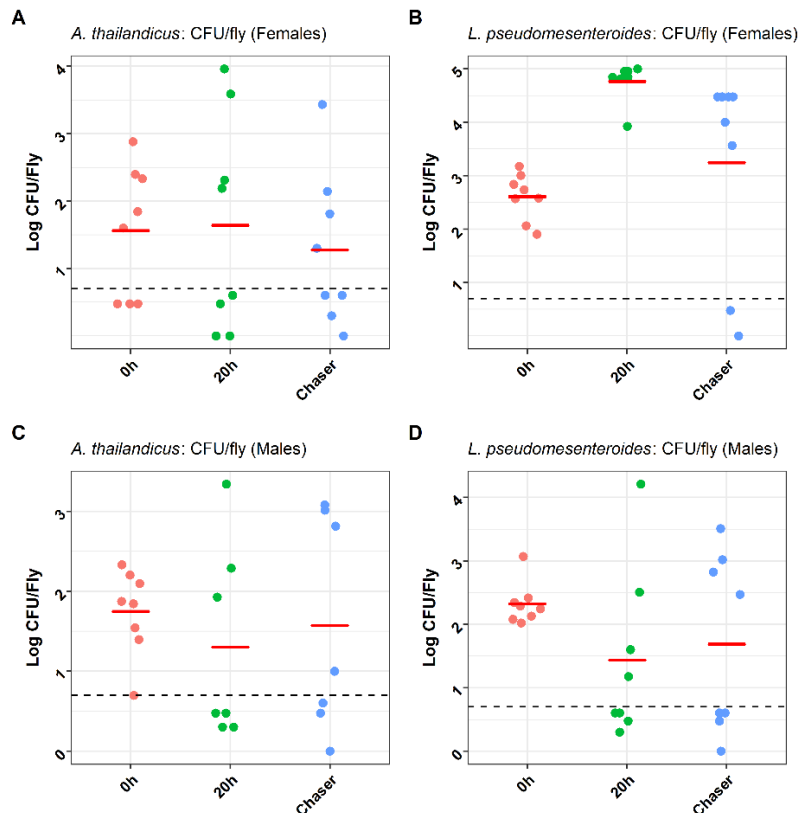


Figure IV.3 - Quantification of CFU/fly after 20 hours of proliferation with chasers. Bacterial levels after 20 hours of proliferation and comparison with chaser flies in female and male flies exposed to *A. thailandicus* (A and C) and *L. pseudomesenteroides* (B and D). Dots and red bar represent a single fly and the mean, respectively. Line shows the minimal detection limit of log CFUs = $\log_{10} 5$.

To mitigate the influence of recontamination of the fly medium, the proliferation time was reduced to 16 hours and levels of *L. pseudomesenteroides* in female and male flies were measured. These results reveal a significant increase (Imm, $p < 0.001$) of bacterial levels in female flies from 0 to 16 hours (1.27 log CFU/fly and 2.29 log CFU/fly) (Figure IV.4A). Simultaneously, chasers' bacterial levels were significantly lower (Imm, $p < 0.001$) from the bacterial levels at 16 hours (1.17 log CFU/fly and 2.29 log CFU/fly, respectively) (Figure 4A). Males, similarly to the previous experiment (Figure IV.3B and IV.3D) *L. pseudomesenteroides* still presented no significant changes ($p > 0.05$) in bacterial levels at 0, 16 hours and chasers (1.53 log CFU/fly; 1.24 log CFU/fly and 0.836 log CFU/fly) (Figure IV.4B).

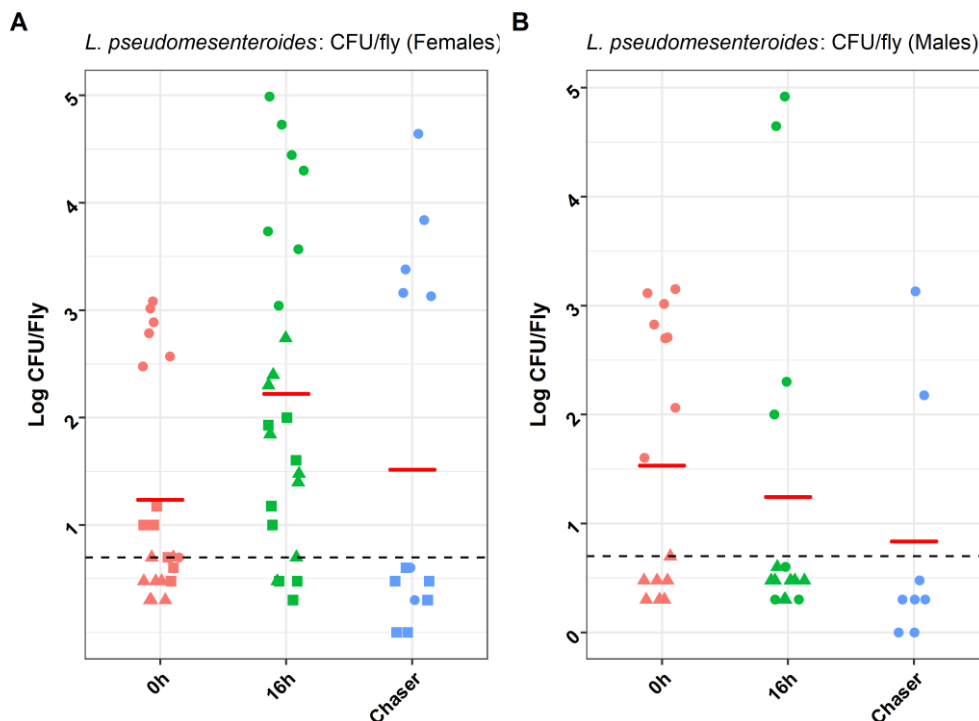


Figure IV.4 - Quantification of CFU/fly after 16 hours of proliferation with chasers (*L. pseudomesenteroides*). Bacterial levels after 16 hours of proliferation and comparison with chaser flies in female (A) and male (B) flies exposed to *L. pseudomesenteroides*. Different shapes represent different replicates. Dots and red bar represent a single fly and the mean, respectively. Line shows the minimal detection limit of log CFUs = $\log_{10} 5$.

A. thailandicus was also tested for 16 hours of proliferation. Unlike *L. pseudomesenteroides*, neither female (Figure IV.5A) or male (Figure IV.5B) flies reveal significantly increased levels (Im, $p > 0.05$) from 0 to 16 hours (0.68 log CFU/fly and 2.48 log CFU/fly; 1.15 log CFU/fly and 1.52 log CFU/fly, respectively) similarly to what was registered for 20 hours (Figure IV.3A and IV.3B).

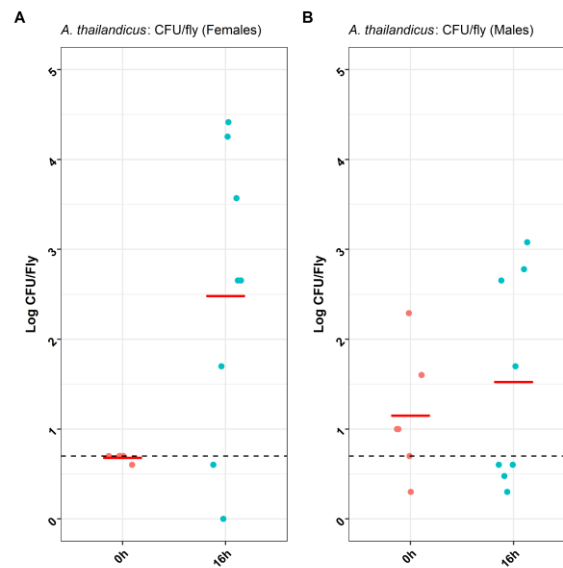


Figure IV.5 - Quantification of CFU/fly after 16 hours of proliferation (*A. thailandicus*). Bacterial levels after 16 hours of proliferation and comparison with chaser flies in female (A) and male (B) flies exposed to *A. thailandicus*. Dots and red bar represent a single fly and the mean, respectively. Line shows the minimal detection limit of log CFUs= $\log_{10} 5$.

3.3. Proliferation of *L. pseudomesenteroides* and *A. thailandicus* in *D. suzukii*: Influence of Sex and Bacterial Strain

Analysis of the previously presented data revealed a different proliferation capacity of *L. pseudomesenteroides* between female and male flies (Figure. IV.4, Imm, $p < 0.01$), confirming a sex-dependent behaviour of this species of bacterium. However, although there is a tendency for the proliferation of *A. thailandicus* in females to be higher than in males (Figure IV.5), the same statistical analysis was not able to find any significant (Im, $p > 0.05$) differences between sexes.

To assess strain-specific differences in proliferation, the proliferation capacity was tested for another isolate, *A. thailandicus* (CP21) in female flies. The levels of bacteria significantly (Im, $p < 0.01$) decreased from 0 to 16 hours (1.669 log CFU/fly and 0.508 log CFU/fly) (Figure IV.6). This resulted in a significant difference (Im, $p < 0.01$) between the levels of bacteria at 16 hours for *A. thailandicus* and *A. thailandicus* (CP21) (2.480 log CFU/fly and 0.508 log CFU/fly), revealing a difference in proliferation capacity of the different isolates.

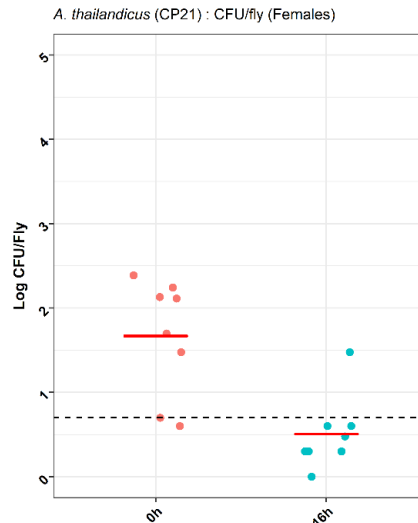


Figure IV.6 - Quantification of CFU/fly after 16 hours of proliferation (*A. thailandicus* (CP21)). Bacterial levels after 16 hours of proliferation and comparison with chaser flies in female flies exposed to *A. thailandicus*. Dots and red bar represent a single fly and the mean, respectively. Line shows the minimal detection limit of log CFUs = $\log_{10} 5$.

4. Discussion

Despite efforts to find new *D. suzukii* control strategies, these still rely heavily on insecticide application, urging to explore innovative and sustainable methods, such as biological control^{20,53,54}. One of the most recent strategies relies on the identification pathogenic microbes, as well as mutualistic ones that may be targeted to induce dysbiosis events in the host and mitigate its damages in agriculture³². The development of novel targeted compounds for bacterial species or (genetic) manipulation for self-killing of the symbiotic microbes has been previously reviewed and theoretically hypothesized, although proven to be a challenge⁵⁵.

It is here presented for the first time, a method to assess the colonizing capacity of bacteria in the gut of *D. suzukii*, based on a methodology established for *D. melanogaster*⁴². *D. suzukii* has been shown to differ in its feeding activity from *D. melanogaster*⁵⁶ and to account for this, different feeding conditions were tested. Consistent bacterial levels at 0 hours were desired to assure that any variation detected after proliferation was due to the proliferation capacity of the bacteria and not due to the lack of ingested bacteria. For *D. suzukii*, 4 hours of feeding prior to overnight starvation produced the most consistent results in both female and male flies.

Once the feeding condition was established, the proliferation time was tested to have an accurate quantification of bacterial proliferation. To achieve this, the assay was performed with chaser (axenic) flies that never contact with the bacteria, so it was possible to take into account for a possible reingestion of the bacteria grown on the fly

medium during the incubation period. In a case where the levels in chaser flies are statistically similar to the levels of the infected flies, no conclusions can be drawn concerning the proliferation ability of the bacteria in the gut. We firstly tested 20 hours of proliferation which, despite enabling a significant increase in bacterial levels of *L. pseudomesenteroides* in female flies, chaser flies also presented significantly higher levels, failing to reject the possibility of recontamination of the fly medium and reingestion of the bacteria. To overcome this issue, the proliferation time was decreased to 16 hours, which significantly decreased the bacterial levels of chaser flies. Thus, proving that the increase in bacterial levels at 16 hours were due to the proliferation capacity of ingested bacteria. This achievement established a final setup, which proves to assess reliably the proliferation capacity of bacteria in the gut of *D. suzukii*. This is a first step to disclose the establishment of a symbiotic relationship between host and microbe.

Most of the bacteria identified in the gut of flies are merely transient, being immediately cleared out after ingestion. The stable populations of bacteria in *Drosophila* spp. are restricted to only a few genera⁵⁷. The biggest challenge in assessing the proliferation capacity of a bacteria in *D. suzukii* was the lack of a positive control. Among the tested species, *L. pseudomesenteroides*, was the one that presented the strongest proliferation capacity in female flies, proving that this method produces reliable and reproducible results and therefore can be used as a positive control. These proliferation results are unexpected, as *L. pseudomesenteroides* was previously identified as detrimental for *D. suzukii*'s survival, possibly falling into the entomopathogen category^{43,58}. However, the identified detrimental strain of *L. pseudomesenteroides* was isolated from moribund larvae⁴³, while the strain used in this study was isolated from an apparently healthy adult female, suggesting that these are two different strains, a virulent and non-virulent one. Considering that there was no proliferation in the gut of male flies, it appears that males can efficiently clear out the bacteria from their intestinal tract, while females provide a colonizing environment for this microorganism. Future work comparing the two different isolates and strains could indicate if a symbiotic link is established and if it is mutualistic or pathogenic, giving a better insight on the role of *L. pseudomesenteroides* in the *D. suzukii* gut.

Interestingly, there was a dichotomous colonizing behaviour of *L. pseudomesenteroides* between the two sexes. The proliferation ability of this bacterium seems to be sex-dependent, as a significant difference was found between male and female flies. Understanding the differences in the microbiota composition of female and

male flies is of the utmost importance to develop novel and more efficient tools of microbial targeting, as it already has been studied with insecticides against *D. suzukii* having a differential efficiency in a sex-dependent way⁵⁹. The physiology between female and male flies is, intuitively, different as they play different roles, namely in reproduction, affecting different parameters of stress response, metabolism, and food intake, as previously described in *D. melanogaster*^{60,61}. Likewise, due to its important role, the microbiome is also sex-dependent, as it differs according to sex in *Drosophila*. In fact, social environments modulate the microbiome structure differentially according to sex in *D. melanogaster*⁶², and the reproductive fitness is dependent on the microbiome state⁶³. In a previous report, the differences in behaviour of female and male *D. suzukii* flies upon elimination of the microbiome were assessed, disclosing a reduced oviposition by axenic female. While axenic males maintained their foraging behaviour, this was reduced in female flies⁴⁸. This could explain the overall more stable and predictable bacterial intake by axenic male flies in comparison to axenic female flies observed in the present study.

Acetobacter spp. are commonly identified bacteria species and known colonizers and modulators of the gut of *Drosophila* spp.^{45,57,64}, and have already been massively identified in *D. suzukii*'s gut^{41,65}. This shows that there is a possible close similarity in the core members of the bacterial community in these genera. *A. thailandicus* was the only species of *Acetobacter* spp. isolated from a wild *D. suzukii* female in this study after 10 days of daily food change, hinting for the stability of this bacteria in its the gut. Previous studies already disclosed this species as a well-established mutualist of *D. melanogaster*, therefore it could potentially be a good candidate to test its colonizing ability in the gut of *D. suzukii*.

As demonstrated by Pais *et al.* (2018) and reviewed by Ma & Leulier (2018), *A. thailandicus* is a gut mutualist of *D. melanogaster*, conferring developmental advantage to flies containing this bacteria species, when compared to axenic or flies carrying a non-colonizing *Acetobacter*^{42,66}. In this study, the bacterial levels at 16 hours in female flies fed with *A. thailandicus* tend to increase in comparison to 0 hours. Now that the method is well-established, further scaled-up studies can be performed to understand if *A. thailandicus* is also a colonizer of *D. suzukii*.

When testing the proliferation potential of a different isolate of *A. thailandicus*, isolated from another female fly of a different region of Portugal (*A. thailandicus* (CP21)), preliminary results show that after 16 hours, there is a clear and significant decay of bacterial levels in female flies and a significantly difference in levels at 16 hours

between the two strains, suggesting a possible strain-specific association of bacteria within a population of *D. suzukii*. More tests are, though, required to thoroughly confirm this pattern.

Although being the same species, the *A. thailandicus* isolated from the original ancestor of the isofemale line used for the proliferation assays shows a tendency to proliferate in the gut of strictly females, while the *A. thailandicus* (CP21), isolated from another population, does not. According to our findings, we observe a possible model of symbiosis establishment between the host (*D. suzukii*) and its mutualistic microbes. This preliminary data suggests that a possible explanation for a sex and strain-specific association of bacteria with *D. suzukii* arises from the contact of a determined population of *D. suzukii* with a community of a strain of a possible symbiotic species, enabling the development of a symbiotic link, naturally selecting more fit bacteria to harbour in their gut. This selection will create specific associations at the sex and strain levels (Figure IV.7).

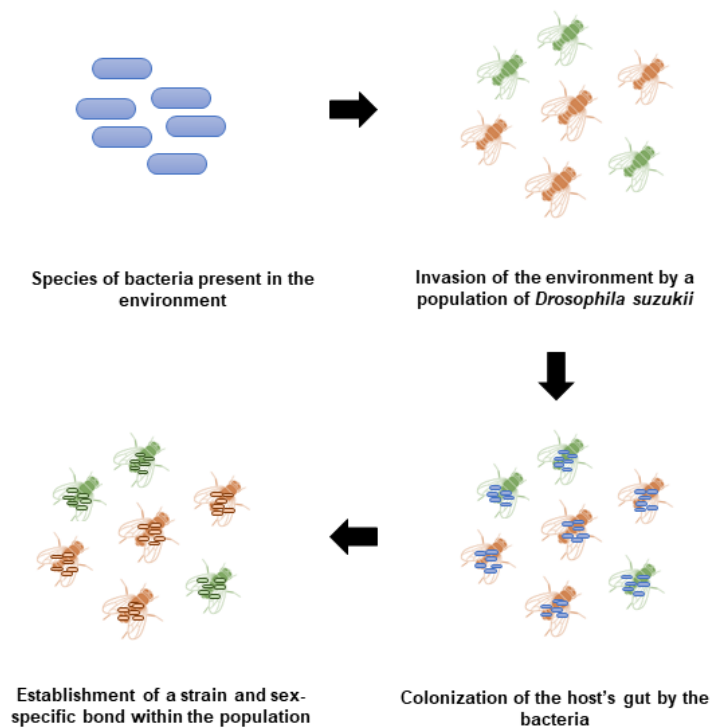


Figure IV.7 - Possible model for the establishment of a strain and sex-specific relationship in *D. suzukii* populations. A colonizing species of bacteria present in the environment (blue rods) is ingested by a population of *D. suzukii* (male and female with different colors) and establishes a symbiotic relationship, which can evolve into a specific strain and sex-dependent link (color match between bacteria and male/female flies).

In conclusion, it was possible to develop a method for the study of the colonizing capacity of naturally isolated bacteria from *D. suzukii*, establishing the right feeding and

proliferation times. This is an important step on understanding the biology and microbe-host interactions of this pest. Also, we detected significant differences between female and male flies, which can have an important impact on the development of novel pest control strategies. Moreover, *D. suzukii* seems to fine-tune its gut microbiota community in a strain-specific way and future work on scaling up these assays should provide further understanding on the microbiota regulation by this pest. New putative colonizers should be tested for the screening of potential targets and pave the way for the development of novel pest control strategies.

References

1. Walsh, D. B. *et al.* *Drosophila suzukii* (Diptera: Drosophilidae): Invasive pest of ripening soft fruit expanding its geographic range and damage potential. *J Integr Pest Manag* **2**, (2011).
2. Kanzawa, T. Studies on *Drosophila suzukii* Mats. *Rev Appl Entomol* **24**, 315 (1936).
3. Kanzawa, T. Studies on *Drosophila suzukii* Mats. *Review of Applied Entomology* **29**, 622 (1939).
4. Asplen, M. K. *et al.* Invasion biology of spotted wing *Drosophila* (*Drosophila suzukii*): a global perspective and future priorities. *J Pest Sci (2004)* **88**, 469–494 (2015).
5. de Ros, G., Grassi, A. & Pantezzi, T. Recent Trends in the Economic Impact of *Drosophila suzukii*. *Drosophila suzukii Management* 11–27 (2020).
6. de Ros, G., Conci, S., Pantezzi, T. & Savini, G. The economic impact of invasive pest *Drosophila suzukii* on berry production in the Province of Trento, Italy. *J Berry Res* **5**, 89–96 (2015).
7. Knapp, L., Mazzi, D. & Finger, R. The economic impact of *Drosophila suzukii*: perceived costs and revenue losses of Swiss cherry, plum and grape growers. *Pest Manag Sci* **77**, 978 (2021).
8. Farnsworth, D. *et al.* Economic analysis of revenue losses and control costs associated with the spotted wing *Drosophila*, *Drosophila suzukii* (Matsumura), in the California raspberry industry. *Pest Manag Sci* **73**, 1083–1090 (2017).
9. Yeh, D. A., Drummond, F. A., Gómez, M. I. & Fan, X. The Economic Impacts and Management of Spotted Wing *Drosophila* (*Drosophila suzukii*): The Case of Wild Blueberries in Maine. *J Econ Entomol* **113**, 1262–1269 (2020).
10. de Ros, G., Anfora, G., Grassi, A. & Ioriatti, C. The potential economic impact of *Drosophila suzukii* on small fruits production in Trentino (Italy). *Integrated protection of fruit crops IOBC-WPRS Bulletin* **91**, 317–321 (2013).
11. Bolda, M. P., Goodhue, R. E. & Zalom, F. G. Spotted Wing *Drosophila*: Potential Economic Impact of a Newly Established Pest. *ARE Update* **13**, 5–8 (2010).
12. Nowak, J. T., McCravy, K. W., Fettig, C. J. & Berisford, C. W. Susceptibility of Adult Hymenopteran Parasitoids of the Nantucket Pine Tip Moth (Lepidoptera: Tortricidae) to

- Broad-Spectrum and Biorational Insecticides in a Laboratory Study. *J. Econ. Entomol* **94**, 1122–1129 (2001).
13. Disi, J. O. & Sial, A. A. Laboratory Selection and Assessment of Resistance Risk in *Drosophila suzukii* (Diptera: Drosophilidae) to Spinosad and Malathion. *Insects* **12**, 794 (2021).
 14. Gress, B. E. & Zalom, F. G. Identification and risk assessment of spinosad resistance in a California population of *Drosophila suzukii*. *Pest Manag Sci* **75**, 1270–1276 (2019).
 15. Haviland, D. R. & Beers, E. H. Chemical Control Programs for *Drosophila suzukii* that Comply With International Limitations on Pesticide Residues for Exported Sweet Cherries. *J Integr Pest Manag* **3**, 1–6 (2012).
 16. Fan, X., Gómez, M. I., Atallah, S. S. & Conrad, J. M. A Bayesian State-Space Approach for Invasive Species Management: The Case of Spotted Wing *Drosophila*. *Am J Agric Econ* **102**, 1227–1244 (2020).
 17. Woltz, J. M., Donahue, K. M., Bruck, D. J. & Lee, J. C. Efficacy of commercially available predators, nematodes and fungal entomopathogens for augmentative control of *Drosophila suzukii*. *Journal of Applied Entomology* **139**, 759–770 (2015).
 18. Haye, T. *et al.* Current SWD IPM tactics and their practical implementation in fruit crops across different regions around the world. *J Pest Sci (2004)* **89**, 643–651 (2016).
 19. Moore, S. D. Biological Control of a Phytosanitary Pest (*Thaumatotibia leucotreta*): A Case Study. *Int J Environ Res Public Health* **18**, 1198 (2021).
 20. Baker, B. P., Green, T. A. & Loker, A. J. Biological control and integrated pest management in organic and conventional systems. *Biological Control* **140**, 104095 (2020).
 21. Wang, X., Daane, K. M., Hoelmer, K. A. & Lee, J. C. Biological Control of Spotted-Wing *Drosophila*: An Update on Promising Agents. *Drosophila suzukii Management* 143–167 (2020).
 22. Mastore, M., Caramella, S., Quadroni, S. & Brivio, M. F. *Drosophila suzukii* Susceptibility to the Oral Administration of *Bacillus thuringiensis*, *Xenorhabdus nematophila* and Its Secondary Metabolites. *Insects* **12**, 635 (2021).
 23. Lacey, L. A. & Goettel, M. S. Current developments in microbial control of insect pests and prospects for the early 21st century. *Entomophaga* **40**, 3–27 (1995).

24. Cossentine, J., Robertson, M. & Xu, D. Biological Activity of *Bacillus thuringiensis* in *Drosophila suzukii* (Diptera: Drosophilidae). *J Econ Entomol* **109**, 1071–1078 (2016).
25. Shower, R., Donati, I., Cellini, A., Spinelli, F. & Mori, N. Insecticidal Activity of *Photorhabdus luminescens* against *Drosophila suzukii*. *Insects* **9**, 148 (2018).
26. Shukla, S. P. & Beran, F. Gut microbiota degrades toxic isothiocyanates in a flea beetle pest. *Mol Ecol* **29**, 4692–4705 (2020).
27. Tagliavia, M., Messina, E., Manachini, B., Cappello, S. & Quatrini, P. The gut microbiota of larvae of *Rhynchophorus ferrugineus* Oliver (Coleoptera: Curculionidae). *BMC Microbiol* **14**, 1–11 (2014).
28. Xia, X. *et al.* Gut microbiota mediate insecticide resistance in the diamondback moth, *Plutella xylostella* (L.). *Front Microbiol* **9**, 25 (2018).
29. Ceja-Navarro, J. A. *et al.* Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. *Nat Commun* **6**, 1–9 (2015).
30. Rudman, S. M. *et al.* Microbiome composition shapes rapid genomic adaptation of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **116**, 20025–20032 (2019).
31. Douglas, A. E. Symbiotic microorganisms: untapped resources for insect pest control. *Trends Biotechnol* **25**, 338–342 (2007).
32. Medina, V. *et al.* Characterized non-transient microbiota from stinkbug (*Nezara viridula*) midgut deactivates soybean chemical defenses. *PLoS One* **13**, e0200161 (2018).
33. Staudacher, H. *et al.* Variability of Bacterial Communities in the Moth *Heliothis virescens* Indicates Transient Association with the Host. *PLoS One* **11**, e0154514 (2016).
34. Ludington, W. B. & Ja, W. W. *Drosophila* as a model for the gut microbiome. *PLoS Pathog* **16**, e1008398 (2020).
35. Trinder, M., Daisley, B. A., Dube, J. S. & Reid, G. *Drosophila melanogaster* as a high-throughput model for host-microbiota interactions. *Front Microbiol* **8**, 751 (2017).
36. Kietz, C., Pollari, V. & Meinander, A. Generating Germ-Free *Drosophila* to Study Gut-Microbe Interactions: Protocol to Rear *Drosophila* Under Axenic Conditions. *Curr Protoc Toxicol* **77**, (2018).
37. Fast, D., Duggal, A. & Foley, E. Monoassociation with *Lactobacillus plantarum* disrupts intestinal homeostasis in adult *Drosophila melanogaster*. *mBio* **9**, e01114-18 (2018).

38. Koyle, M. L. *et al.* Rearing the Fruit Fly *Drosophila melanogaster* Under Axenic and Gnotobiotic Conditions. *JoVE (Journal of Visualized Experiments)* e54219 (2016).
39. Bing, X., Gerlach, J., Loeb, G. & Buchon, N. Nutrient-Dependent Impact of Microbes on *Drosophila suzukii* Development. *mBio* **9**, e02199-17 (2018).
40. Solomon, G. M., Dodangoda, H., McCarthy-Walker, T. T., Ntim-Gyakari, R. R. & Newell, P. D. The microbiota of *Drosophila suzukii* influences the larval development of *Drosophila melanogaster*. *PeerJ* **7**, e8097 (2019).
41. Pais, I. S., Valente, R. S., Sporniak, M. & Teixeira, L. *Drosophila melanogaster* establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. *PLoS Biol* **16**, e2005710 (2018).
42. Hiebert, N., Carrau, T., Bartling, M., Vilcinskas, A. & Lee, K. Z. Identification of entomopathogenic bacteria associated with the invasive pest *Drosophila suzukii* in infested areas of Germany. *J Invertebr Pathol* **173**, 1–11 (2020).
43. Jiménez-Padilla, Y., Esan, E. O., Floate, K. D. & Sinclair, B. J. Persistence of diet effects on the microbiota of *Drosophila suzukii* (Diptera: Drosophilidae). *Canadian Entomologist* **152**, 516–531 (2020).
44. Lin, Q. *et al.* Analyses of the gut bacteriomes of four important *Drosophila* pests. *Canadian Entomologist* **153**, 757–773 (2021).
45. Martínez-Sañudo, I. *et al.* Metagenomic analysis reveals changes of the *Drosophila suzukii* microbiota in the newly colonized regions. *Insect Sci* **25**, 833–846 (2018).
46. Chandler, J. A., James, P. M., Jospin, G. & Lang, J. M. The bacterial communities of *Drosophila suzukii* collected from undamaged cherries. *PeerJ* **2**, e474 (2014).
47. Shu, R. *et al.* Sex-Dependent Effects of the Microbiome on Foraging and Locomotion in *Drosophila suzukii*. *Front Microbiol* **12**, 656406 (2021).
48. R Core Team. R: A Language and Environment for Statistical Computing. Preprint at <https://www.R-project.org/> (2020).
49. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models Using lme4. *J Stat Softw* **67**, 1–48 (2015).
50. Lenth, R. v. emmeans: Estimated Marginal Means, aka Least-Squares Means. Preprint at <https://CRAN.R-project.org/package=emmeans> (2022).

51. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (Springer-Verlag New York, 2016).
52. Girod, P. *et al.* Host specificity of Asian parasitoids for potential classical biological control of *Drosophila suzukii*. *J Pest Sci* (2004) **91**, 1241–1250 (2018).
53. Wang, X. Biological control of *Drosophila suzukii*. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* **15**, (2020).
54. Darby, A. C. Symbiosis research as a novel strategy for insect pest control. *Biorational Control of Arthropod Pests: Application and Resistance Management* 207–231 (2009).
55. Lin, Q. C. *et al.* Behavioral Rhythms of *Drosophila suzukii* and *Drosophila melanogaster*. *Florida Entomologist* **97**, 1424–1433 (2014).
56. Broderick, N. A. & Lemaitre, B. Gut-associated microbes of *Drosophila melanogaster*. *Gut Microbes* **3**, 307–321 (2012).
57. Hiebert, N. *et al.* The Gram-Positive Bacterium *Leuconostoc pseudomesenteroides* Shows Insecticidal Activity against Drosophilid and Aphid Pests. *Insects* **11**, 1–15 (2020).
58. Smirle, M. J., Zurowski, C. L., Ayyanath, M. M., Scott, I. M. & MacKenzie, K. E. Laboratory studies of insecticide efficacy and resistance in *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) populations from British Columbia, Canada. *Pest Manag Sci* **73**, 130–137 (2017).
59. Chandegra, B., Tang, J. L. Y., Chi, H. & Alic, N. Sexually dimorphic effects of dietary sugar on lifespan, feeding and starvation resistance in *Drosophila*. *Aging* **9**, 2521–2528 (2017).
60. Sieber, M. H. & Spradling, A. C. Steroid Signaling Establishes a Female Metabolic State and Regulates SREBP to Control Oocyte Lipid Accumulation. *Current Biology* **25**, 993–1004 (2015).
61. Leech, T. *et al.* Social environment drives sex and age-specific variation in *Drosophila melanogaster* microbiome composition and predicted function. *Mol Ecol* **30**, 5831–5843 (2021).
62. Morimoto, J., Simpson, S. J. & Ponton, F. Direct and trans-generational effects of male and female gut microbiota in *Drosophila melanogaster*. *Biol Lett* **13**, (2017).

63. Shin, S. C. *et al.* *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science* (1979) **334**, 670–674 (2011).
64. Vacchini, V. *et al.* Bacterial diversity shift determined by different diets in the gut of the spotted wing fly *Drosophila suzukii* is primarily reflected on acetic acid bacteria. *Environ Microbiol Rep* **9**, 91–103 (2017).
65. Ma, D. & Leulier, F. The importance of being persistent: The first true resident gut symbiont in *Drosophila*. *PLoS Biol* **16**, e2006945 (2018).

V. Chapter 5: General Discussion

Red fruits and berries producers around the world face the challenging task to control and mitigate the serious damages of the pest *Drosophila suzukii*. This species was first identified in East Asia in the first half of the previous century^{1,2}, and since 2008 it has invaded the European and American continents³⁻⁵. *D. suzukii* is present in Portugal since 2012, when it was first identified in the regions of Odemira and Algarve, swiftly spreading towards the North region of the country⁶. Currently, producers rely on the application of pesticides to mitigate the presence and damage of *D. suzukii*^{7,8}, which not only has economic implications for the acquisition of products, equipment, and personnel⁹⁻¹², but also environmental and public health costs, which may lead to long-term consequences of hard repair¹³⁻¹⁶.

To establish new strategies of control and Integrated Pest Management (IPM) programs, biological control strategies are in the current scope of research as they present environmental and economic advantages over the use of pesticides¹⁷⁻²⁰. Among the different strategies of biocontrol, the manipulation of the microbiome and the use of entomopathogenic bacteria is of great interest, due to the important role of the gut microbiome in homeostasis maintenance and behaviour control²¹⁻²⁵.

This dissertation aimed to contribute to filling the gap in knowledge regarding *D. suzukii* bacteriome and harness the potential of associated bacteria as entomopathogens or symbionts to develop new strategies for pest control. To achieve this, *D. suzukii* samples were collected from different orchards in the North region of Portugal and their microbiome was analysed by metagenomics and isolation of bacteria. Furthermore, the isolated bacteria were used to build a library of gut-associated microbes with *D. suzukii* and test either the entomopathogenic or symbiotic potential of some of the isolated bacteria towards this pest.

This discussion will dwell on the main observations reported on the previous II, III and IV chapters of this dissertation and how they contribute to the understanding of *D. suzukii* biology and the development of new biocontrol strategies.

1. Characterization of the Microbiome of Portuguese *Drosophila suzukii* Populations

Chapter II aimed to characterize the microbiota associated with populations of *D. suzukii* from different orchards. This was the first step to understanding the diversity and plasticity of the microbiome in comparison to both different populations in Portugal

(and to other countries) and different conditions (wild and fruit-emerged). After the analysis of the microbiome, the Proteobacteria phylum was prominent, corroborating previous results^{26–28}. The abundance of these bacteria in *D. suzukii* suggests a tendency to the structure of its microbiome.

Acetobacteraceae was identified as the most abundant family of the core microbiota. This observation supports, not only previous observations of the association of these bacteria with *Drosophila* spp.^{29–31}, but also sustains the association with *D. suzukii* in particular^{32,33}. Establishing a core microbiome provides important data about the structure of the microbial community that has important physiological associations with *Drosophila* spp.³⁴. Thus, the previous data is important to find important targets for microbiota disruption, as it was approached in Chapter IV.

Furthermore, the isolation of microorganisms showed that the *Tatumella* genus may, also be part of the core microbial community of this species, particularly, in larvae taking into account that it has been previously identified as one of the most abundant genera in *D. suzukii* larvae^{26,32}. Here, we corroborated these observations as *Tatumella* was isolated mainly from larvae, suggesting an important association with these bacteria. This finding has implications on the plasticity of the microbiome according to the stage of development, which could ultimately help in the development of strategies that mainly target specific stages of development, particularly because the larval stage is the most problematic as it causes damages in ripen fruits.

Moreover, the observed prominence of the *Frigoribacterium* genus among the different field-collected samples was a surprising result as this is the first report of its identification among *Drosophila* spp. Unfortunately, there is little to no characterization of this genus regarding its role as a microbe associated with insects, although it has been identified in other species of insects^{35–37}. This observation may suggest an important member of the microbial community of Portuguese *D. suzukii* populations, establishing a specific relationship with Portuguese genotypes. If this is the case, this could provide a proof-of-concept of how the characterization of the microbiome of different populations is fine-tuned. Moreover, this may provide a starting point for the development of new strategies to disrupt the microbiome of Portuguese populations of *D. suzukii*.

Besides analysing the bacterial community associated with wild *D. suzukii* to obtain an important overview of the bacteria associated with the host in a natural environment, understanding the shaping factors of the bacterial community of *D. suzukii* was also a goal of this study. Although we were able to find distanced samples from raspberry and

blueberry-emerged individuals, females and males also distanced in the diversity of bacteria, allowing no correlation to be established between diet and sex as shaping factors. Nevertheless, these conditions have been previously reported to affect microbiome composition in *D. melanogaster*^{38,39}. So, to further confirm the impact in *D. suzukii*, larger samples are needed for a more robust statistical inference. Furthermore, Chapters III and IV suggest a sex-dependent response to different bacteria, whether it is regarding infection or symbiosis.

The characterization of the microbiota of Portuguese populations of *D. suzukii* provided in this dissertation is a necessary step on the development of biocontrol tools as we need to deepen the knowledge on the structure and foundation of the bacterial communities present in the fly to then look at it as a manipulable structure. This work provided a detailed description of bacteria identified by employing metagenomic and isolation tools. It stresses the importance of employing metagenomic tools as much information may be lost due to the obstacles of artificial isolation of microorganisms that may be acquired only by molecular approaches. Nevertheless, to assess the functional role of bacteria, isolation of these bacteria must be accomplished, to further explore and find new possible natural pathogens or symbionts.

2. Sex-Dependent Response of *Drosophila suzukii* to Bacterial Infections

Previous studies have searched for natural entomopathogenic bacteria towards *D. suzukii*^{26,40}. In Chapter III, we took advantage of the isolated bacteria in Chapter II to test their entomopathogenic potential towards *D. suzukii* flies. We aimed to characterize their detrimental activity at two levels, at a macro level by evaluating insecticidal activity and at the molecular immune level, employing RT-qPCR to quantify transcript expression of immune response genes, namely from the Imd and Toll pathways, that generally play an important role against infections⁴¹.

Oral infection with two putative entomopathogenic bacteria, *Microbacterium thalassium* and *Providencia* sp., were revealed to not affect mortality for the first 6 days after infection. Although revealing poor insecticidal activity in a short span, other parameters could have been affected, such as fertility or development. To understand if there were any mechanisms of resistance upon oral infection with these bacteria, molecular pathways of immune response were studied. This was the first report in which Imd and Toll pathways have been fully studied in *D. suzukii*, namely their target genes, *duox*,

dptA, and *drs* (which encode for Dual-oxidase, Diptericin, and Drosomycin), which play an important part in the regulation of microbiota and bacterial infections^{41,42}.

Despite revealing no significant differential expression of antimicrobial peptides (AMPs) upon infection, *duox* was differentially regulated according to exposure time in female *D. suzukii* and according to bacteria in males. This suggests that similarly to other *Drosophila* spp., the Dual-oxidase also responds as an important gut microbiota regulator and as a first-line of response⁴². Moreover, delayed activation of the Toll pathway in males was observed, which is also congruent with previous reports for *D. melanogaster*⁴¹. Hence, the first descriptions of molecular immune pathways dynamics were obtained for *D. suzukii*.

Principal Component Analysis (PCA) was employed to further disclose molecular patterns to explain mechanisms of resistance to infection with the two bacteria. A sex-dependent response to infection was observed as female *D. suzukii* disclosed more sensitivity to *Providencia* sp. infection, tending to activate the Imd pathway and release Diptericin. Contrarily, male flies displayed more sensitivity to *M. thalassium* and focused on the late activation of the Toll pathway. This is also a pioneer study on the influence of sex in the response to putative pathogens, which we consider an important parameter as it may influence the performance of a given pesticide or biocontrol agent⁴³.

These results disclose important insights into the immune molecular mechanisms of *D. suzukii* and how they are differentially regulated in the two sexes. Understanding these molecular mechanisms may open new doors on how to overcome these resistance mechanisms and molecularly engineer bacteria in order to escape the immune response of *D. suzukii*. So, further studies should employ the quantification of these and other immune and stress response molecular pathways to describe the dynamics and biology of *D. suzukii* upon infections to develop more effective biocontrol agents.

3. Methods to Study Symbiotic Bacteria in *Drosophila suzukii*

Although entomopathogenic bacteria remain the focus of research to develop biocontrol tools, the search for symbionts and mutualistic bacteria should not be ignored as it may provide important insight to develop strategies of microbiome disruption to induce dysbiosis and, therefore, detrimental effects⁴⁴. In Chapter IV, a method to study symbiotic bacteria of *D. suzukii* was optimized based on a previous method established for *D. melanogaster*⁴⁵. The proliferation capacity in the gut of *D.*

suzukii of *Acetobacter thailandicus* and *Leuconostoc pseudomesenteroides* isolates was assessed.

The optimization of the protocol for the evaluation of the proliferation capacity of bacteria in the gut of *D. suzukii* resulted in a method that produces reliable and reproducible results. An important outcome of the development of this method was the establishment of a positive control for the proliferation in female *D. suzukii*, *L. pseudomesenteroides*. This provides a well-established model for further experiments with other bacteria. Besides, this work also revealed sex-dependent proliferation of the same bacteria: *L. pseudomesenteroides* colonizes the gut of female flies but not of male flies. Once again, as discussed in Chapter III, this is an important parameter that must be considered and is sometimes overlooked. When developing strategies for microbiome manipulation, this can have a great impact on the efficiency of control tools.

Besides a sex-dependent proliferation displayed by *L. pseudomesenteroides*, the results also disclosed a strain-specific proliferation capacity of *A. thailandicus*. Although *A. thailandicus* did not reveal significant proliferation in the gut of *D. suzukii*, when another isolate of the same species, from another sample of another region, was used, statistical analysis revealed a significantly different behaviour, suggesting an establishment of a strain-specific link with *D. suzukii*. This observation may influence further research of symbiotic and possible molecular targets as microbial communities from different *D. suzukii* populations may differ in their microbiome at the strain level.

Integrating this preliminary data, we propose a model in which a certain population of *D. suzukii* upon invasion of a new habitat is colonized by a strain of colonizing bacteria. This established relationship will then be fine-tuned after generations and become tightly specific to both the bacterial strain and sex.

4. Final Remarks

In summary, the work developed throughout this dissertation period and explored in the previous chapters followed the proposed objectives and released pioneer studies on *D. suzukii* and information of Portuguese samples of this pest.

The characterization of the microbial communities of Portuguese samples of *D. suzukii* has been now disclosed and sets a first step towards describing the core microbiome of Portuguese *D. suzukii* populations. Moreover, this work also assessed the entomopathogenic potential of two naturally isolated bacteria and disclosed the

molecular mechanisms of resistance of this pest for the first time and, parallelly developed a method for the study of colonizing bacteria in *D. suzukii*.

In conclusion, this work provides transversal research by harnessing the whole potential of the microbiome as a tool to develop new pest control strategies, leading to more efficient and cost-beneficial alternatives to current control practices. This would, ultimately, have a positive impact in the movement towards a more a more resilient and sustainable primary production system.

V.II. Future Perspectives

This dissertation is an exploratory study and provided pioneer assays towards the understanding of the *D. suzukii* pest and development of biocontrol and sustainable strategies. However, some scientific questions were raised and are of great importance to achieve the main goal.

- a) **Validate the characterization of the microbiome of Portuguese populations of *D. suzukii*.** To thoroughly describe the core microbiome of Portuguese populations of *D. suzukii*, other regions of the country should be considered and studied in order to validate our findings and possibly to establish other species of bacteria specific to distinct areas. This will enable a thorough understanding of the structure and dynamics of the microbial community associated with *D. suzukii* and screen possible targets for biocontrol.
- b) **Test new putative entomopathogenic and symbiotic bacteria.** Although this study tested two putative entomopathogenic bacteria and two putative symbiotic bacteria, the library of naturally isolated bacteria is much more extensive allowing for the study of different bacteria regarding their entomopathogenic or symbiotic potential.
- c) **Explore sex-dependent immune response.** The search for effective biocontrol tools relies heavily on disclosing parameters that may affect their effectiveness. This study revealed different resistance molecular responses between sexes, which must be further explored, for example, by extending research to other immune and stress response pathways and also by establishing *D. suzukii* mutants for the different pathways to evaluate susceptibility changes.
- d) **Understand the dynamics of strain-specific proliferation.** To elaborate deeper on our findings, isolates from different regions may be tested for their proliferation capacity and across different generations to test the hypothesis proposed in our model in Chapter IV.

These questions still remain unanswered and are of great importance for the scientific community to dwell on them and for the agroindustry to obtain new solutions and IPM-sustainable strategies for pest control.

References

1. Kanzawa, T. Studies on *Drosophila suzukii* Mats. *Rev Appl Entomol* **24**, 315 (1936).
2. Kanzawa, T. Studies on *Drosophila suzukii* Mats. *Review of Applied Entomology* **29**, 622 (1939).
3. Calabria, G., Máca, J., Bächli, G., Serra, L. & Pascual, M. First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. *Journal of Applied Entomology* **136**, 139–147 (2012).
4. Hauser M, Gaimari S & Damus M. *Drosophila suzukii* new to North America. *Fly times* **43**, 12–15 (2009).
5. Deprá, M., Poppe, J. L., Schmitz, H. J., de Toni, D. C. & Valente, V. L. S. The first records of the invasive pest *Drosophila suzukii* in the South American continent. *J Pest Sci (2004)* **87**, 379–383 (2014).
6. Franco, A. M. *Drosophila suzukii* chegou a Portugal com ataque à framboesa. Sanidade. Pequenos frutos. *Voz do Campo* **158**, 36–37 (2013).
7. van Timmeren, S. & Isaacs, R. Control of spotted wing *Drosophila*, *Drosophila suzukii*, by specific insecticides and by conventional and organic crop protection programs. *Crop Protection* **54**, 126–133 (2013).
8. Sial, A. A. *et al.* Evaluation of organic insecticides for management of spotted-wing *Drosophila* (*Drosophila suzukii*) in berry crops. *Journal of Applied Entomology* **143**, 593–608 (2019).
9. de Ros, G., Grassi, A. & Pantezzi, T. Recent Trends in the Economic Impact of *Drosophila suzukii*. *Drosophila suzukii Management* 11–27 (2020).
10. Bolda, M. P., Goodhue, R. E. & Zalom, F. G. Spotted Wing *Drosophila*: Potential Economic Impact of a Newly Established Pest. *ARE Update* **13**, 5–8 (2010).
11. Pimentel, D. Environmental and Economic Costs of the Application of Pesticides Primarily in the United States. *Environ Dev Sustain* **7**, 229–252 (2005).
12. Farnsworth, D. *et al.* Economic analysis of revenue losses and control costs associated with the spotted wing *Drosophila*, *Drosophila suzukii* (Matsumura), in the California raspberry industry. *Pest Manag Sci* **73**, 1083–1090 (2017).
13. Sookhtanlou, M., Allahyari, M. S. & Surujlal, J. Health Risk of Potato Farmers Exposed to Overuse of Chemical Pesticides in Iran. *Saf Health Work* **13**, 23–31 (2022).

14. Iwasaki, J. M. & Hogendoorn, K. Non-insecticide pesticide impacts on bees: A review of methods and reported outcomes. *Agric Ecosyst Environ* **314**, 107423 (2021).
15. Sun, S., Zhang, C. & Hu, R. Determinants and overuse of pesticides in grain production: A comparison of rice, maize and wheat in China. *China Agricultural Economic Review* **12**, 367–379 (2020).
16. Haviland, D. R. & Beers, E. H. Chemical Control Programs for *Drosophila suzukii* that Comply With International Limitations on Pesticide Residues for Exported Sweet Cherries. *J Integr Pest Manag* **3**, 1–6 (2012).
17. Lacey, L. A., Frutos, R., Kaya, H. K. & Vail, P. Insect Pathogens as Biological Control Agents: Do They Have a Future? *Biological Control* **21**, 230–248 (2001).
18. Baker, B. P., Green, T. A. & Loker, A. J. Biological control and integrated pest management in organic and conventional systems. *Biological Control* **140**, 104095 (2020).
19. Ruijter, L. Insect Pathogenic Bacteria in Integrated Pest Management. *Insects* **6**, 352–367 (2015).
20. Tait, G. et al. *Drosophila suzukii* (Diptera: Drosophilidae): A Decade of Research Towards a Sustainable Integrated Pest Management Program. *J Econ Entomol* **114**, 1950–1974 (2021).
21. Gould, A. L. et al. Microbiome interactions shape host fitness. *Proc Natl Acad Sci U S A* **115**, E11951–E11960 (2018).
22. Weiss, B. & Aksoy, S. Microbiome influences on insect host vector competence. *Trends Parasitol* **27**, 514–522 (2011).
23. Jia, Y. et al. Gut microbiome modulates *Drosophila* aggression through octopamine signaling. *Nat Commun* **12**, 1–12 (2021).
24. McMullen, J. G., Peters-Schulze, G., Cai, J., Patterson, A. D. & Douglas, A. E. How gut microbiome interactions affect nutritional traits of *Drosophila melanogaster*. *J Exp Biol* **223**, jeb227843 (2020).
25. Muñoz-Benavent, M., Pérez-Cobas, A. E., García-Ferris, C., Moya, A. & Latorre, A. Insects' potential: Understanding the functional role of their gut microbiome. *J Pharm Biomed Anal* **194**, (2021).

26. Hiebert, N., Carrau, T., Bartling, M., Vilcinskas, A. & Lee, K. Z. Identification of entomopathogenic bacteria associated with the invasive pest *Drosophila suzukii* in infested areas of Germany. *J Invertebr Pathol* **173**, 1–11 (2020).
27. Jiménez-Padilla, Y., Esan, E. O., Floate, K. D. & Sinclair, B. J. Persistence of diet effects on the microbiota of *Drosophila suzukii* (Diptera: Drosophilidae). *Canadian Entomologist* **152**, 516–531 (2020).
28. Lin, Q. *et al.* Analyses of the gut bacteriomes of four important *Drosophila* pests. *Canadian Entomologist* **153**, 757–773 (2021).
29. Crotti, E. *et al.* Acetic acid bacteria, newly emerging symbionts of insects. *Appl Environ Microbiol* **76**, 6963–6970 (2010).
30. Broderick, N. A. & Lemaitre, B. Gut-associated microbes of *Drosophila melanogaster*. *Gut Microbes* **3**, 307–321 (2012).
31. Adair, K. L., Wilson, M., Bost, A. & Douglas, A. E. Microbial community assembly in wild populations of the fruit fly *Drosophila melanogaster*. *ISME J* **12**, 959–972 (2018).
32. Chandler, J. A., James, P. M., Jospin, G. & Lang, J. M. The bacterial communities of *Drosophila suzukii* collected from undamaged cherries. *PeerJ* **2**, e474 (2014).
33. Vacchini, V. *et al.* Bacterial diversity shift determined by different diets in the gut of the spotted wing fly *Drosophila suzukii* is primarily reflected on acetic acid bacteria. *Environ Microbiol Rep* **9**, 91–103 (2017).
34. Staubach, F., Baines, J. F., Künzel, S., Bik, E. M. & Petrov, D. A. Host Species and Environmental Effects on Bacterial Communities Associated with *Drosophila* in the Laboratory and in the Natural Environment. *PLoS One* **8**, e70749 (2013).
35. Khan, S., Somerville, D., Frese, M. & Nayudu, M. Environmental gut bacteria in European honey bees (*Apis mellifera*) from Australia and their relationship to the chalkbrood disease. *PLoS One* **15**, e0238252 (2020).
36. He, C., Nan, X., Zhang, Z. & Li, M. Composition and diversity analysis of the gut bacterial community of the oriental armyworm, *Mythimna separata*, determined by culture-independent and culture-dependent techniques. *Journal of Insect Science* **13**, 165 (2013).
37. Adams, A. S., Boone, C. K., Bohlmann, J. & Raffa, K. F. Responses of Bark Beetle-Associated Bacteria to Host Monoterpenes and Their Relationship to Insect Life Histories. *J Chem Ecol* **37**, 808–817 (2011).

38. Leech, T. *et al.* Social environment drives sex and age-specific variation in *Drosophila melanogaster* microbiome composition and predicted function. *Mol Ecol* **30**, 5831–5843 (2021).
39. Jehrke, L., Stewart, F. A., Droste, A. & Beller, M. The impact of genome variation and diet on the metabolic phenotype and microbiome composition of *Drosophila melanogaster*. *Sci Rep* **8**, 1–15 (2018).
40. Bing, X. L., Winkler, J., Gerlach, J., Loeb, G. & Buchon, N. Identification of natural pathogens from wild *Drosophila suzukii*. *Pest Manag Sci* **77**, 1594–1606 (2021).
41. de Gregorio, E., Spellman, P. T., Tzou, P., Rubin, G. M. & Lemaitre, B. The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *EMBO J* **21**, 2568–2579 (2002).
42. Kim, S. H. & Lee, W. J. Role of DUOX in gut inflammation: Lessons from *Drosophila* model of gut-microbiota interactions. *Front Cell Infect Microbiol* **3**, 116 (2014).
43. Andreazza, F. *et al.* Sex-dependent locomotion and physiological responses shape the insecticidal susceptibility of parasitoid wasps. *Environmental Pollution* **264**, 114605 (2020).
44. Douglas, A. E. Symbiotic microorganisms: untapped resources for insect pest control. *Trends Biotechnol* **25**, 338–342 (2007).
45. Pais, I. S., Valente, R. S., Sporniak, M. & Teixeira, L. *Drosophila melanogaster* establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. *PLoS Biol* **16**, e2005710 (2018).