Universidade do Minho
Escola de Engenharia

Henrique Gonçalves Ribeiro

Novel phage-derived product to control *Paenibacillus larvae* in bee hives

setembro de 2022

Novel phage-derived product to control *Paenibacillus larvae* in bee hives
Henrique Gonçalves Ribeiro
UMinho | 2022

Universidade do Minho
Escola de Engenharia

Henrique Gonçalves Ribeiro

Novel phage-derived product to control *Paenibacillus larvae* in bee hives

Dissertation for Ph.D. degree in

Chemical and Biological Engineering

Work Supervised by:

Ana Oliveira (Ph. D.)

And Co-supervised by:

Professor Joana Azeredo

September 2022

ACKNOWLEDGEMENTS

Com o final desta jornada, não poderei deixar de agradecer a todos os que fizeram parte dela e contribuíram de forma especial para a minha vida. Por isso, um muito obrigado a todos por me acompanharem neste desafio e me ajudarem na realização desta tese.

Quero agradecer em primeiro lugar, à Ana Oliveira, minha orientadora e amiga, por ter acreditado e confiado em mim, pelo constante apoio e desafio que me proporcionou no desenvolvimento deste projeto. Obrigado por me conduzires na ciência, há bem mais tempo do que este doutoramento de 4 anos e uns meses de desconto, alcançando um trabalho do qual podemos ficar orgulhosos para o Futuro. Juntos superamos muitos desafios que me trouxeram até aqui e marcarão a minha forma de estar na ciência e na vida, (sim consegui ler os livros todos da Saga Millennium).

Gostaria de agradecer à Professora Joana Azeredo, pela sua coorientação neste projeto, pela forma entusiasmada que nos faz acreditar que haverá uma solução positiva e promissora. Obrigado por me deixar fazer parte da sua equipa e do seu grupo de trabalho o BBiG (Bacteriophage Biotechnology Group), permitindo a realização deste trabalho com todas as condições e apoios.

I would also like to thank Danish Malik for the opportunity he gave me to go and do a little internship in his laboratory, which due to the circumstances was short but in the future anything can happen.

Quero deixar um abraço e uma palavra de agradecimento ao Tiago Moreira da Bee Prado pela disponibilidade para nos ajudar nos ensaios no campo com os seus apiários e as suas abelhas.

Aos meus colegas e amigos que fazem ou já fizeram parte do LPhage e BBiG um obrigado por todas os momentos de aprendizagem, de discussão, de desabafo, de diversão e parvoíce que tivemos, tornou a viagem muito mais satisfatória. Quero deixar uma palavra especial ao Luís, a Priscila, ao Sílvio, ao Hugo, a Diana, a Rita Costa, e a Catarina pelo seu envolvimento e contributo ao longo do tempo no trabalho bem como as conversas e troca de ideias que tivemos. Aos meus amigos e parceiros de aventura e de laboratório um obrigado por terem a coragem de entrarem neste barco comigo e seguirmos viagem com as alegrias e tristezas uns dos outros e partilharmos a nossa ajuda, a Alice, Ana Brandão, Daniela Araújo, Daniela Silva, Ergun, Graça, Luciana, Rodrigo, Rute, Susana, Tânia e Vânia. Aos meus amigos do CEB pelas conversas de motivação de corredor, de cantina e do lanche, Beatriz, Carla, Daniel, Débora, Diana, Michel, Márcia, Óscar, Renato. Aos meus amigos de curso, Luís, Carlos, Bruno, João Carlos, João Pacheco e Tânia pelas conversas e constante boa disposição.

Não posso deixar de agradecer a Ana Teresa, por teres aparecido neste momento da minha vida e pelo constante apoio durante esta reta final do doutoramento.

À minha família, a minha avó Augusta, a minha mãe, ao meu pai, ao meu irmão e ao meu tio, a minha madrinha Rosalina, os meus primos e priminhos um obrigado por serem a minha família, por em alegrarem, por me apoiarem e darem força para ir para a frente.

Por último, mas também muito importante, quero deixar um forte abraço ao meu avô Artur que não conseguiu ver-me a iniciar este caminho, mas certamente me guiou nele, e um bom beijo as minhas tias Laurinda e Maria que me viram iniciar o caminho, mas não chegaram ao final dele comigo. Está sempre presente o vosso amor, e sinto-me acompanhado por vocês todos os dias. Esta tese é dedicada a vocês.

As bolsas que me permitiram a elaboração deste trabalho de dissertação foram financiadas pela Fundação Ciência e Tecnologia (FCT) tendo as seguintes referências: SFRH/BD/128859/2017 e COVID/BD/151856/2021, para o qual deixo o meu agradecimento.

Este estudo foi financiado pelo projeto APILYSE, PTDC/CVT-EPI/4008/2014 - POCI-01-0145-FEDER-016598, - financiado pelo FEDER através do COMPETE 2020 - Programa Operacional Competitividade e Internacionalização (POCI) e por fundos nacionais através da FCT. O trabalho contou ainda com o financiamento estratégico da unidade UID/BIO/04469/2013, UID/BIO/04469/2019 e UIDB/04469/2020, COMPETE 2020 (POCI-01-0145-FEDER-006684) e operação BioTecNorte (NORTE-01-0145-FEDER-000004), financiado pelo Fundo Europeu de Desenvolvimento Regional no âmbito do Norte2020 - Programa Operacional Regional do Norte.

"Se fizeres obras impactantes, tens sempre o teu espaço. Não queiras viver com as tuas medalhas, estão lá atrás." – Valete 2019

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

Novo produto derivado de fagos para controlar a *Paenibacillus larvae* em colmeias de abelhas

Loque Americana é uma doença bacteriana causada pela *Paenibacillus larvae*, resultando na morte das larvas de abelha, tendo um enorme impacto negativo na apicultura. Atualmente, na Europa, não existem terapias disponíveis e a queima de colónias infetadas é obrigatória. Os bacteriófagos (fagos), vírus que infetam especificamente as bactérias, causando a sua lise e consequente eliminação (biocontrolo), são candidatos promissores para reduzir o impacto da doença nas colmeias. O principal objetivo deste trabalho foi o desenvolvimento de uma formulação à base de fagos que, após ser administrada às abelhas adultas, é entregue por regurgitação às larvas. A biodistribução do fago nas colmeias, foi efetuada utilizando o fago T7 como modelo. Após a administração de partículas virais nos alimentadores das abelhas, a sua viabilidade foi avaliada através da formação de placas de inibição (PFU), e a deteção do DNA fagico por PCR quantitativo. Apesar de se terem encontrado fagos viáveis nos tecidos larvares, a maior parte não estava viável, sugerindo a importância de proteger os fagos do ambiente da colmeia. Novos fagos de *P. larvae* foram isolados, caracterizados e três deles selecionados: vB_PlaP_API480, o primeiro podovírus detetado capaz de infetar estirpes de *P. larvae*, e dois siphovírus, vB_PlaS_API46 e vB_PlaS_API59. A taxa de infeção do API480 (73%) foi maior do que as outras (34% e 62% respectivamente), sendo selecionado para ser avaliado *in vivo*. A biodistribução do API480 nas abelhas adultas e larvas foi estudada, em colmeias saudáveis, para avaliar o potencial de encapsulamento dos fagos. Apesar de permitir que os fagos atinjam as larvas, a formulação de fago encapsulado não foi bem dispersa no alimento das abelhas e deve ser melhorada. Usando o mesmo fago em larvas infetadas em laboratório, este revelou ser seguro para as larvas e com efeitos antibacterianos *in vivo*, particularmente quando as larvas foram subsequentemente tratadas com um outro fago (vB_PlaS_CEB16), ativo contra mutantes resistentes ao API480. No estudo do impacto dos profagos na evolução da virulência e fitness de *P. larvae*, pode observar-se que a maioria das estirpes abrigam profagos intactos que influenciam o seu desempenho, e novos *clusters* e *singletons* de fagos de *P. larvae* foram identificados nos alinhamentos dos genomas dos seus hospedeiros. No geral, este trabalho avalia a administração oral de fagos para as abelhas adultas, em formulações encapsuladas, para controlar mais eficientemente a *P. larvae* nas colmeias, contribuindo também para aumentar o estado da arte da população de fagos de *P. larvae* e as suas características genómicas e taxonómicas.

Palavras-chave: Bacteriófago, Larvas de abelhas, Loque Americana, Controlo antibacteriano, *Paenibacillus larvae*

ABSTRACT

Novel phage-derived product to control *Paenibacillus larvae* in bee hives

American Foulbrood (AFB) is a bacterial disease caused by *Paenibacillus larvae*, resulting in the death of bee larvae, having a huge negative impact on beekeeping. Currently, in Europe, there are no therapies available and the burning of infected colonies is mandatory. Bacteriophages (phages), viruses that specifically infect bacteria, causing their lysis and consequent elimination (biocontrol), are hopeful candidates to reduce the impact of this disease in hives. The main objective of this work was the development of a phage-based formulation that, after being administered to adult bees, is delivered by regurgitation to larvae. The phage biodistribution in hives was established using the T7 phage as a model. After the administration of viral particles in bee feeders, their viability was assessed through the formation of inhibition plates (PFU), and the detection of phage DNA by quantitative PCR. Despite viable phages being found in larval tissues, most of them were not viable, suggesting the importance of protecting phages from the hive environment. New *P. larvae* phages were isolated, characterized and three of them selected: vB_PlaP_API480, the firstly podovirus detected able to infect *P. larvae* strains, and two siphovirus, vB_PlaS_API46 and vB_PlaS_API59. The infection rate of API480 (73%) was higher than the others (34% and 62% respectively) and was selected to be evaluated *in vivo*. The biodistribution of API480 in adult bees and larvae tissues was studied in healthy hives to evaluate the potential of phage encapsulation. Despite allowing phages to reach larvae, the encapsulated phage formulation was not well dispersed in bee food and must be improved. Using the same phage on infected lab-reared larvae, proved to be safe for larvae and with *in vivo* antibacterial effects, particularly when larvae were subsequently treated with another phage (vB_PlaS_CEB16), active against API480-resistant mutants. In the study of the impact of prophages in the evolution of *P. larvae* virulence and fitness, it could be observed that most strains harbour intact prophages that influence their performance, and new clusters and singletons of *P. larvae* phages were identified in the genome alignments of their hosts. Overall, this work evaluates the oral administration of phages to adult bees, in encapsulated formulations, to control *P. larvae* more efficiently in hives, also contributing to increase the state of the art of *P. larvae* phages population and their genomic and taxonomic characteristics.

Keywords: Bacteriophage, Bee larvae, American Foulbrood, Antibacterial control, *Paenibacillus larvae*

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations, Acronyms and Symbols

aa – Amino Acid
ABC transporter – ATP-Binding Cassette transporter
Abd – Abdomens
AFB – American Foulbrood Disease
AMR – Antimicrobial Resistance Genes
ANOVA – Analysis of variance
BCCM – Belgian Coordinated Collections of Microorganisms
BLASTn – Basic Local Alignment Search Tool to nucleotide
BLASTp – Basic Local Alignment Search Tool to protein
BLIP – β-Lactamase Inhibitory Proteins
bp – Base pair
BRED – Bacteriophage Recombineering of Electroporated DNA
BSA – Bovine Serum Albumin
C1 to C22 – Clusters
C1 B – Control group of lab-reared larvae, baseline without spores or phages
C2 S – Control group of lab-reared larvae, with only spores
C3 P – Control group of lab-reared larvae, with only phages
CARD – The Comprehensive Antibiotic Resistance Database
CBD – Cell Binding Domain
CCUG – Culture Collection of the Goteborg University
CDS – Coding Sequences
CECT – Colección Española de Cultivos Tipo
CFU – Colony Forming Units
COG – Cluster of Orthologous Groups
Cq – Quantification cycle
CRISPR – Clustured Regularly Interspaced Short Palindromic Repeat
DNA – Deoxyribonucleic acid
dsDNA – Double stranded DNA
DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
dUTP pyrophosphatase – Deoxyuridine triphosphate pyrophosphatase
EAD – Enzymatic (or Catalytic) Active Domain
EFB – European Foulbrood
EOP – Efficiency of Plating
ERIC – Enterobacterial Repetitive Intergenic Consensus
ESI-MS/MS – Electrospray Ionization Mass Spectrometry/Mass Spectrometry
EtxB – Epsilon-Toxin type B
EU – European Union
FAO – Food and Agriculture Organization of the United Nations
Fe-S – Iron–sulfur
FITC – Fluorescein isothiocyanate
G1 – Hive fed with API480 phage encapsulated in Eudragit S100
G2 – Hive fed with API480 phage in a free form
GbpA – N-acetylglucosamine (GlcNAc)-binding protein A
GC content – Guanine and Cytosine content
gp – Gene product

H&T – Heads and Thoraxes' content
HGT – Horizontal Gene Transfer
IAC – Internal Amplification Control
ICTV – International Committee on Taxonomy of Viruses
K_2SO_4 – Potassium sulfate
kDa – kilo Dalton
LFW – Lysis From Without
LIN – Lincomycin
LMG – Laboratory of Microbiology of Ghent University
LOD – Limit of Detection
MBL – Metal β-Lactamase
Mbp – Megabase pair
MCP – Major Capsid Protein gene
MFS – Major Facilitator Superfamily transporter
MHG – Mid- Hindgut
MOI – Multiplicity of Infection
mRNA – Messenger Ribosomal Ribonucleic Acid
MRS broth – Man, Rogosa & Sharpe broth
MTP – Major Tail Protein
MYPGP agar – Mueller-Hinton Broth, Yeast extract, Potassium hydrogen phosphate, Glucose and Sodium-pyruvate
N/A – Non-applicable
NCBI – National Center for Biotechnology Information
O/N – Overnight
OD – Optical Density
OSGC – One-Step Growth Curve
OTC – Oxytetracycline
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PES – Polyethersulfone filters
PFU – Plaque Forming Unit
PgpA – Phosphatidylglycerophosphatase A
PHASTER – PHAge Search Tool Enhanced Release software
PM – Peritrophic Matrix
PVL – Panton-Valentine Leukocidin (leukotoxin) system
qPCR – Quantitative Polymerase Chain Reaction
RGI – Resistance Gene Identifier
RJ – Royal Jelly
RNA – Ribonucleic Acid
rpm – Rotations per minute
rRNA – Ribosomal Ribonucleic Acid
RT – Room Temperature
S1 to S12 – Singletons
ScpB – Segregation and Condensation Protein B
SD – Standard Deviations
SDB – Sabouraud Dextrose Broth
SDS-PAGE – Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
SM Buffer – Saline Magnesium Buffer

SMR – Small Multidrug Resistance transporter
SPG – Shirasu Porous Glass
TA system – Toxin-Antitoxin system
TAP – Tail Assembly Protein
TBST – Tris Buffered Saline with 0.1% (v/v) Tween 20 1 ×
TEM – Transmission Electron Microscopy
TetR – Tetracycline Resistance
TMP – Tail Tape Measure Protein
TRITC – Tetramethylrhodamine
TSA – Tryptic Soy Agar medium
TSB – Tryptic Soy Broth medium
TSP – Tail Sheath Protein
Tt1 – First phage treatment tested, single dose of API480 phage
Tt2 – Second phage treatment tested, double dose of API480 phage
Tt3 – Third phage treatment tested, first dose of API480 and the second dose of CEB16
TTP – Tail Tube Protein
TTSS – Type Three (III) Secretion System
TYL – Tylosin
UB – Universal Buffer
Wt – Wild type
Yops – *Yersinia* outer proteins

SCIENTIFIC OUTPUTS / LIST OF PUBLICATIONS

Papers in peer reviewed journals (*first author):

Ribeiro HG*, Nilsson A, Melo LDR, Oliveira A. **Analysis of intact prophages in genomes of *Paenibacillus larvae*: An important pathogen for bees.** *Front Microbiol.* 2022;13 (July). https://doi.org/10.3389/fmicb.2022.903861

Ribeiro HG*, Melo LDR, Oliveira H, et al. **Characterization of a new podovirus infecting *Paenibacillus larvae*.** *Sci Rep*. 2019;9(1):1-12. https://doi.org/10.1038/s41598-019-56699-y

Ribeiro HG*, Correia R, Moreira T, Vilas Boas D, Azeredo J, Oliveira A. **Bacteriophage biodistribution and infectivity from honeybee to bee larvae using a T7 phage model.** *Sci Rep*. 2019;9(1):620. https://doi.org/10.1038/s41598-018-36432-x

Other papers in peer reviewed journals:

Fernandes L*, Ribeiro H, Oliveira A, et al. **Portuguese honeys as antimicrobial agents against *Candida* species.** *J Tradit Complement Med*. 2020. https://doi.org/10.1016/j.jtcme.2020.02.007

Posters in conferences:

Ribeiro HG*, Nilsson A, Melo LDR, Oliveira A. **Can integrated prophages affect virulence and fitness of the honeybee pathogen *Paenibacillus larvae*?** Viruses of Microbes 2022, 18-22 July 2022.

Ribeiro HG*, Nilsson A, Azeredo J, Melo LDR, Oliveira A. **Role and impact of prophages in *Paenibacillus larvae*.** Phage Futures Congress 2021, 24-26 February 2021. (Online)

Ribeiro HG*, Melo LDR, Boon M, Lavigne R, Azeredo J, Oliveira A. **API480: features towards therapy in honeybee hives.** 23rd Biennial Evergreen International Phage Meeting, Olympia, WA - The Evergreen State College, 4-9 August 2019.

Oral communications:

Ribeiro HG*, Azeredo J, Oliveira A. **Development of bacteriophage solutions to fight against American Foulbrood Disease**. Biofilms CEB Seminar, Braga, Portugal, November 9th, 2021.

Ribeiro HG*, Azeredo J, Oliveira A. **Bacteriophage preparation to prevent and control American Foulbrood Disease – Oral delivery.** 1st Chemical and Biological Engineering Doctoral Symposium (1st CBEDS), Braga, Portugal, September 16-18, 2020. (Online)

Participation in roundtable session "Investigação vs Indústria: Onde se encontram?", IX Conference of Biochemistry at the University of Minho (10 April 2018)

Works contributing to oral communications:

Ribeiro HG, Oliveira A*. **Phage therapy in Apiculture – Prospects.** Viruses of Microbes 2020 (iVoM) – Webinar series: Take a walk on the VoM side, January 21, 2021. (Online) https://www.youtube.com/watch?v=kH7x_M09yp4

Ribeiro HG, Correia R, Moreira T, Vilas Boas D, Azeredo J, Oliveira A*. **Bacteriophage biodistribution and infectivity from honeybee to bee larvae.** Viruses of Microbes 2018 (EMBO Workshop), Wroclaw, Poland, July 9-13, 2018.

STRUCTURE OF THE THESIS

This thesis is divided into seven chapters, describing the scientific work developed.

Chapter 1 is the literature review of the topics addressed throughout this thesis. This chapter first approaches the role and lifestyle of honeybees, followed by the importance and relevance of American Foulbrood disease (AFB) and their bacterial pathogen *Paenibacillus larvae*. Lastly, the state of the art of bacteriophage traits, focusing on overview of *P. larvae* phages diversity, as well the phage therapy studies reported to overcome this bacterium.

Chapter 2 is an *in vivo* study where healthy hives were used to assess the biodistribution and bioavailability of a phage model in adult bee and young larvae tissues after oral feeding.

Chapter 3 describes the isolation and characterization (genome annotation, morphologic analysis and pH stability) of first podovirus *P. larvae* phage (vB_PlaP_API480). The lytic spectra, phage growth parameters and stability in hive conditions, are good indicators supporting their potential use in AFB biocontrol.

In **Chapter 4** the isolation and characterization (genome annotation, and morphologic analysis) of two new siphovirus *P. larvae* phages (vB_PlaS_API46 and vB_PlaS_API59) is described.

Chapter 5 reports an *in vivo* study where healthy hives were used to assess the biodistribution and bioavailability of *P. larvae* phage encapsulated and free phage after oral feeding. Also, details an *in vivo* study with lab-reared larvae where time-lapsed phage administration with different phages were applied to evaluate the prevention and control of AFB (reduce *P. larvae* load inside infected lab-reared larvae).

Chapter 6 is an *in-silico* study of *P. larvae* prophages found in *P. larvae* genomes. These prophages were analysed relatively to the presence of virulence, toxic and antimicrobial resistance genes, as well to others genes that might confer advantages to their bacterial hosts fitness.

Finally, in **Chapter 7**, the major conclusions reached during this scientific work are summarized. Additionally, future research development is the scope of this thesis is suggested.

CHAPTER 1: LITERATURE REVIEW

1.1 Apiculture and the environmental role of honeybees

Beekeeping is an ancestral activity, carried out since the beginnings of Human civilization, in the Egyptian, Greek and Roman empires for example (Radcliffe & Seeley, 2021). Nowadays, the EU is the second-largest honey producer in the world after China, with 12% and 24% of all world production respectively, where around 615000 beekeepers manage 18.9 million colonies and produce 280000 tonnes of honey per year, generating more than 400 million euros per year (European Commission, 2019, 2021).

All these numbers in modern beekeeping are possible mainly through the species of honeybees *Apis mellifera*. These are less aggressive species of bees, producing a good yield of honey and easily forming new swarms at low costs. These small insects classified as arthropods (invertebrate animals having an exoskeleton) play an interesting and important role in the environment. Being responsible for most of pollination of commercial crops and wild plants worldwide, they have a strong impact on the economy of the sector and biodiversity in general. In fact, the Food and Agriculture Organization of the United Nations (FAO) projects that 71 of the 100 crop species responsible for 90% of food worldwide are pollinated by bees. The natural nectar collection process for making honey has consecrated bees as the most important pollinating insects for ecosystems, having a huge economic impact on agriculture (Genersch, 2010a; R. Johnson & Lynne Corn, 2016; Yue, Nordhoff, Wieler, & Genersch, 2008). Bee activities provide a variety of products, like honey, royal jelly (RJ), propolis, bee pollen and bee wax, that are used in diverse industries such as food and diet supplantation, natural medicine, pharmacology, and cosmetology. Nevertheless, the number of bees and colonies that disappear are increasing by the action of diverse factors like, parasites, pathogens (bacterial, viral and fungal) (FAO, 2006; Genersch, 2010a, 2010b), pesticides and poor nutrition (Corby-Harris et al., 2020; Deboutte et al., 2020).

1.2 Honeybees' social organization, larvae life cycle, bee anatomy and microbiome

Honeybees are considered superorganisms that form colonies in hives and lives as eusocial organization. There are several social status (reproductive division of labour), cooperative brood care and intersection of generations (Radcliffe, 2021). Inside one hive, in average are 50000 individuals, with different ages and specialized function that cooperate to live in a group. In hives there is a queen, dozens of drones (reproductive castes female and males respectively) and thousands of works bees (non-reproductive female caste) (**Figure 1**). The queen lays the eggs (in the peak production period produces 1500-2000 eggs/day) that are dropped in the bottom of the cell comb. The drones mate with queens

from other hives to change genetically the new offspring (Radcliffe, 2021). The worker bees perform lots of different jobs depending on their age. For example, the young workers also known as nurse bees, take care of the queen and brood (eggs, larvae and pupa) by feeding them and keep the hive clean. The middle-aged adults build and repair the nest with wax, protect the hive from invaders and produce honey. The older workers have the riskier tasks like leaving the hive to collect the resources needed to make the hive's food: flower nectar and pollen (B. R. Johnson, 2010; Radcliffe, 2021).

Figure 1: Different honeybee castes: queen, worker and drone. Removed from Youtube video: Beekeeping - Honey Bee Caste (Accessed https://www.youtube.com/watch?v=esJXJS4G3FM accessed on 30 November 2021).

The three types of castes pass through the stage of egg, larva and pupa before becoming adults. After three days the eggs hatches to larvae, which evolves in different instars, for five to seven days. Then, until the cells are capped, starting the pro-pupa and pupa stages. Usually, the period of growth is 16, 21 or 24 days for queens, workers, or drones respectively (Gurung, M. B., Partap, U., Sharma, H. K., Islam, N and Tamag, 2012).

The hypopharyngeal glands located on head of worker bees are responsible for the production of RJ, a protein-rich secretion that will feed first-instars worker bee larvae or queen bee larvae for all stages. It is also composed by the a lipid-rich secretion produced in the mandibular glands and by nectar or diluted honey added from the bee crop (Oliver, 2021a, 2021b). RJ is a complex acid mixture (pH 3.4–4.5) with enzymes, phenolic compounds, mineral salts and vitamins, that has several proprieties like antimicrobial, antiviral and antiseptic for example that is also explored to human applications (Barnutiu, Marghitas, Dezmirean, Mihai, & Bobis, 2011; Chen, 1995; Pasupuleti, Sammugam, Ramesh, & Gan, 2017).

Organized in colonies, bees have their own defence mechanisms conferring social immunity, which are dependent on the behaviour of all the individuals of the group. This mechanism of defences has several actions, for example the hygienic behaviour, the social fever or the use of propolis, an antimicrobial

compound made by plant resins, allows to reduce or eliminate exposure to pathogens or pest infestation inside the hives (Evans et al., 2006; Simone-Finstrom, 2017).

Adult bees are divided on three sections: head, thorax and abdomen. Focusing on the digestive system, the hypopharyngeal glands are in the head, the thorax is crossed by the esophagus that goes from the mouth (head) until the crop or "honey/nectar stomach" (abdomen). Then, also in the abdomen, there are the midgut organs, where the digestion occurs, like ventriculus, the hindgut organs as ileum (where nutrients are absorbed) and the rectum (which stores the waste) (Carreck et al., 2013; Chan et al., 2013; Faux, 2021) (**Figure 2**). The pH levels of adult bee guts are low in the crop (pH 4.5–5.5), rising in the midgut (pH 5.6–7.0), and lowering again in the hindgut (pH 5.2–6.3) (Colibar, Popovici, Eugeniu, & Korodi, 2010; Zheng, Powell, Steele, Dietrich, & Moran, 2017).

The gut microbiome of honeybees is very conserved and stable, playing several roles in their health, as conferring protection to pathogen infections or influencing metabolism and food digestion. This specialized microbial community is acquired by oral trophallaxis, fecal-oral expose and contact with hive material and is not found outside of the bee gut or hive environment (Kwong & Moran, 2016; Powell, Martinson, Urban-Mead, & Moran, 2014). These food-sharing mechanisms are so nearest that the honeybee colony is often regarded as having a "social stomach", since in few hours the fresh ingested nectar or water are spread to a higher number of individuals in the hive (K. Crailsheim, 1990a; Karl Crailsheim, 1992; DeGrandi-Hoffman & Hagler, 2000; Nicolson, 2009; Nixon & Ribbands, 1952).

Although adult bees emerge with few or without any gut microbial flora (during the metamorphosis the gut lining is shed), all are colonized in the first days (Kwong & Moran, 2016; Powell et al., 2014; Vojvodic, Rehan, & Anderson, 2013). The core microbiome found comprises five bacterial species, *Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus* sp. Firm 5, *Lactobacillus* sp. Firm 4, and *Bifidobacterium* spp. Less prevalently but also found are the non-core bacterium *Frischella perrara*, *Bartonella apis* and *Parasaccharibacter apium* (**Figure 2**). These bacteria are 99.9% of the microbiome present in the honeybee gut and typically colonize a specific digestive tissue. While the hindgut sections are densely colonized (10^8 – 10^9 bacterial cells), the crop and the midgut sections are usually unfavourable to bacterial survival (Kwong & Moran, 2016; Powell et al., 2014). The species present and their dominance in the intestinal microbiome of bees can change according to season, age, caste, tasks and diet. The bee microbiome reflects the health status of the hive and any unbalance caused by agrochemicals (pesticides), or antibiotics is a concern. Atypical opportunistic bacteria species such as *Serratia*, *Klebsiella*, *Hafnia* and *Enterobacter* can be identified changing the common microbiota (Kwong & Moran, 2016; Raymann & Moran, 2018).

Main pests and diseases of the hive are caused by: i) bacteria like *Paenibacillus larvae* that cause the American Foulbrood disease and *Melissococcus plutonius* source de European Foulbrood disease; ii) fungus such as *Nosema* spp. responsible for Nosema disease or *Ascosphaera apis* origin of Chalkbrood disease; iii) viruses as Deformed Wing Virus or Sacbrood Virus for example; iv) parasites like the *Varroa destructor* or *Acarapis woodi* (FAO, 2006; Genersch, 2010a, 2010b).

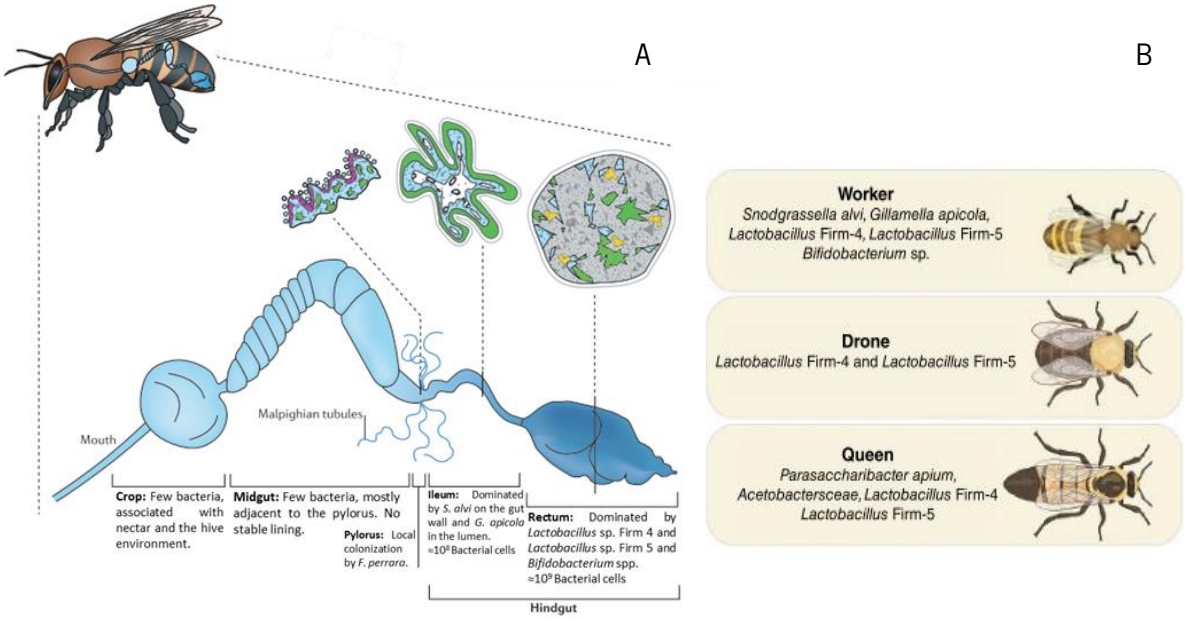

Figure 2: Distribution of microbiome in guts of adult bees castes. (A) Composition and spatial organization of bacterial microbiome in the worker bee gut. (B) The prevalent commensal bacteria found in the gut of workers, drones, and queens. Adapted from Kwong & Moran, 2016; Raymann, 2021.

1.3 American Foulbrood Disease (AFB): an overview of the threat

One of the most devastating bacterial diseases listed by the World Organisation for Animal Health – Office International des Epizooties (OIE 2020), is the American Foulbrood (AFB) (Genersch, 2010a). Despite its name, this pathology is not confined to the American continent. AFB is caused by *Paenibacillus larvae*, a worldwide-distributed spore-forming bacterium. It is Gram-positive, rod-shaped (0.5 µm wide and 1.5–6 µm long) and facultative anaerobe. *P. larvae* spores are highly contagious and highly resistant, as they can remain dormant for more than 35–50 years under cold, heat, humidity and drought, and easily spread across apiaries (Bassi et al., 2015; Genersch, 2006; Genersch, Ashiralieva, & Fries, 2005; Heyndrickx et al., 1996). This bacterium affects bee brood, and only in the initial stages of development (first 36 hours after egg hatching) (Karl Crailsheim & Riessberger-Gallé, 2001; Genersch, 2010a; Genersch et al., 2005; Woodrow, 1942), while adult bees are resistant to the infection, accumulating the spores in their organs (Wilson, 1971). If no immediate actions are taken to combat AFB, the disease spreads within the colony and may reach healthy hives in the apiary due to robbing, drifting workers or

cross-contamination caused by beekeeper manipulations (Bassi et al., 2015; Genersch, 2010a; Rauch, Ashiralieva, Hedtke, & Genersch, 2009).

The contamination initiates when nurse bees, the vehicle to spread spores, feed young larvae with *P. larvae*-contaminated food. The presence of 10 spores inside first-instar larvae are enough to initiate AFB, leading to hive collapse and outbreaks (**Figure 3**) (Genersch et al., 2005; Woodrow, 1942; Woodrow & Holst, 1942). The resistance of larvae to *P. larvae* strains is age dependent. The digestive system of older larvae, is more mature and complex and the peritrophic matrix (PM) is thicker, creating more difficulties for bacteria to penetrate (Garcia-Gonzalez & Genersch, 2013; Yue et al., 2008). In sensitive larvae, spore germination seems to occur 12 hours after spore ingestion, randomly along midgut lumen (Yue et al., 2008). Then, vegetative bacteria proliferate massively before they start to locally rupture the epithelium and quickly invade the haemocoel. Despite sporulation occurs mainly in the final stage of infection, where less nutrients are available, it is also verified all along all the invasion process (**Figure 4A**). In the end, the larvae biomass is transformed in a brownish ropy mass, semi-fluid, glue-like colloid, releasing a putrid smell, forming scales when dried very difficult to remove by honeybees (**Figure 4B**). The germination of spores in the midgut of young larvae is possible due to: i) intestinal pH levels of 6.8 (Colibar et al., 2010), within ideal pH range observed *in vitro*; ii) temperature of 35 °C, reached by the heat produced by the bees; iii) specific conditions, that do not occur in older larvae or adult bees, such as the presence of uric acid (a waste product of the metabolic breakdown of purines and proteins with nitrogen) in the same compartment as L-tyrosine (an amino acid obtained by proteins degradation) (Alvarado, Phui, Elekonich, & Abel-Santos, 2013).

In vitro, on complex media, only about 10% of the *P. larvae* spores plated seem to germinate on vegetative cells (Alvarado et al., 2013; Forsgren, Stevanovic, & Fries, 2008). Moreover, the acid environment of products found in hive such as honey, pollen, RJ and nectar between pH 3.0–4.5, seems to prevent the germination of *P. larvae* spores in a places other than larvae midgut lumen (Alvarado et al., 2013).

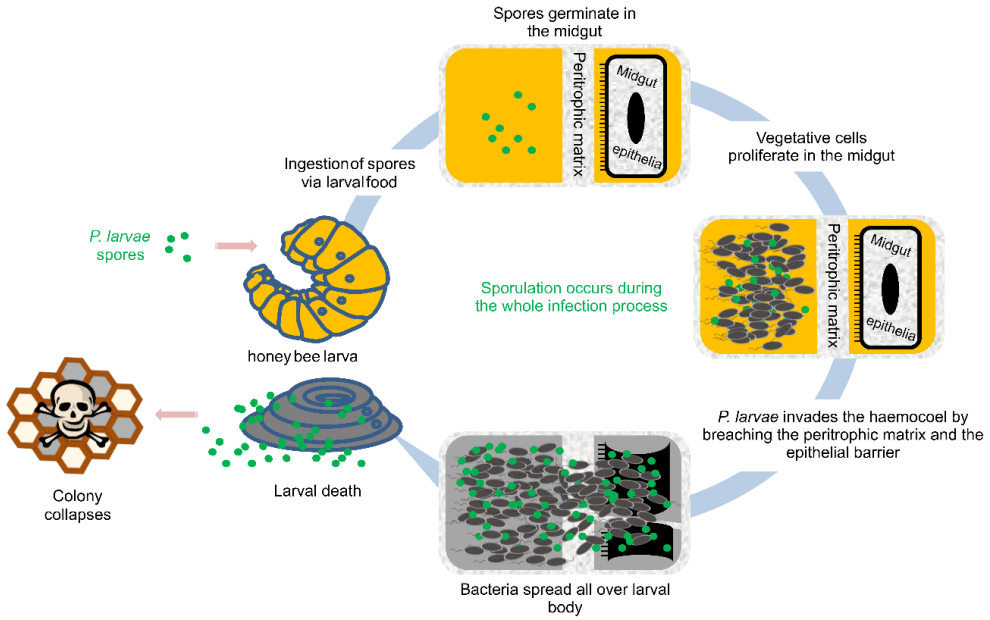

Figure 3: *Paenibacillus larvae* infection model, the bacterial life cycle in infected larvae. Adapted from Djukic et al., 2014.

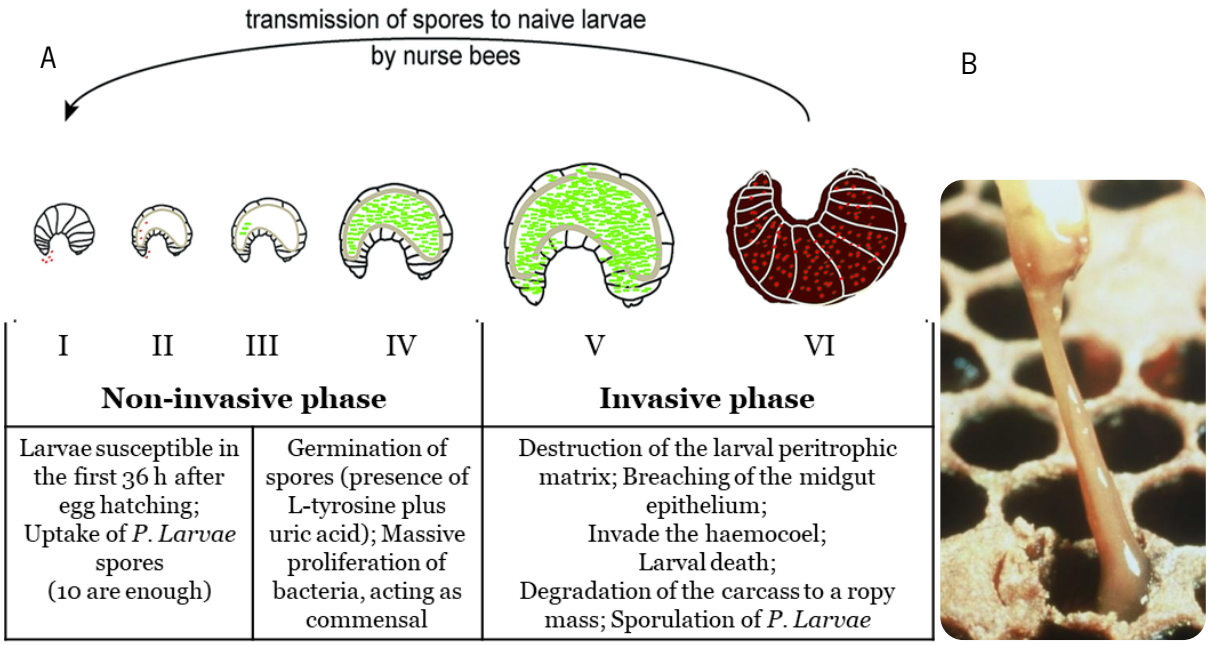

Figure 4: Descriptive pathogenesis of American Foulbrood disease. (A) The non-invasive phase of infections comprises the ingestion of *P. larvae* spores by naive larvae through spore contaminated larval diet (I–II). The spores germinate in the larval midgut (III) where the vegetative bacteria proliferate massively until they occupy nearly the entire midgut lumen (IV). The invasive phase of infection is initiated by the destruction of the midgut peritrophic matrix enabling the bacteria to attack and cross the epithelial barrier (V). By then the infected larva is dead and *P. larvae* totally degrade the cadaver to a ropy mass before bacterial sporulation occurs. The ropy mass consisting of billions of spores dries down to the so called 'foulbrood scale'. Nurse bees trying to clean the brood cell become contaminated by these spores and transmit them to naive larvae when feeding them and the next infection cycle begins. Red dots - spores; Green ovals - vegetative bacteria. Adapted from Müller et al., 2015; Poppinga & Genersch, 2015. (B) Ropy mass of a larvae in a brood comb region. Classical method of AFB detection with a toothpick.

1.4 *Paenibacillus larvae* diversity, virulence and biology

P. larvae species can be subdivided into five different genotypes based on amplification Enterobacterial Repetitive Intergenic Consensus (ERIC): ERIC I, II, III, IV and V (Beims et al., 2020; Genersch, 2006; Genersch & Otten, 2003). The main studies involving *P. larvae* have been performed with ERIC I and II, the most significant genotypes responsible for most AFB outbreaks (Biová et al., 2021; Genersch, 2010a; Žugelj et al., 2021). No ERIC III and IV strains have been isolated from field outbreaks in recent years, existing only a few strains in culture collections. Recently a strain belonging to the new genotype ERIC V was isolated and studied in detail (Beims et al., 2020).

The severity of the AFB varies with the genotype involved in the infection. For example, larvae infected with ERIC I die within 12 to 14 days (slow killer strains), the ERIC II cause the death of the larvae after six to seven days (medium fast killer strains) and ERIC III–V, more virulent genotypes, cause the larvae death just in three days (fast killer strains) (Beims et al., 2020). Previous studies indicated that ERIC III and ERIC IV needed about seven days to kill 100% of the infected hosts (Ashiralieva & Genersch, 2006; Genersch, 2006; Genersch et al., 2005; Rauch et al., 2009). Despite ERIC I is classified as the less virulent genotype to larvae, this is the more aggressive with infections resulting in higher load of spores in the hive, with longer effect (Beims et al., 2020; Rauch et al., 2009). On causing a slow death to larvae, some die in comb cells which are already capped, their efficient spore removal from the hive by nurse bees through the hygienic behaviour is delayed. Around 40% of infected larvae may remain in the hive after 13 days, forming millions of new of *P. larvae* spores after its death (Rauch et al., 2009). Concerning ERIC II, larvae die more rapidly, enabling nurse bees to detect and remove them quicky from the hive (about 86% of the infected larvae are removed in the first five days, achieving 90% after 13 days post-infection). The social immunity obtained after ERIC II infections reflects a smaller number of new spores available within the hive, since only 10% of the diseased larvae seems to remain there (Rauch et al., 2009).

Depending on the genotypes, *P. larvae* shows phenotypic and metabolic differences. For example, ERIC I and ERIC IV mainly produce non-pigmented colonies while ERIC II and III are yellow to orange pigmented depending on the cultivation in solid media (Antúnez et al., 2007; Bassi et al., 2015; Genersch, 2006; Heyndrickx et al., 1996; Neuendorf, Hedtke, Tangen, & Genersch, 2004). No data is available about ERIC V colony traits. ERIC I and ERIC II strains have opposite positions relative to metabolism of some sugars, while ERIC I strains, metabolize trehalose, some glucose, but none of them fructose, ERIC II strains metabolize fructose, most of them trehalose and some glucose (Bassi et al., 2015; Genersch, 2006; Neuendorf et al., 2004). The spores ERIC III-V share the same morphology, having a rough surface,

with ridges evident along the spores, while ERIC I and ERIC II spores have smooth surfaces (Beims et al., 2020).

Besides phenotypic and metabolic differences, several virulence factors depends on ERIC genotype. The ability to rupture and penetrate PM through degradation of its main constituent, N-acetyl-D-glucosamine, is caused by the chitin-degrading enzyme *Pl*CBP49, common to all five ERIC genotypes (Beims et al., 2020; Erban et al., 2019; Garcia-Gonzalez, Poppinga, et al., 2014). This is a very important enzyme to pathogenesis of *P. larvae* permitting the transition from the non-invasive to the invasive phase (**Figure 5**). In 2012, the S-layer protein SplA, was identified and reported as being an ERIC II specific protein, with function to promote the bacterial adhesion to the midgut epithelium (Fünfhaus & Genersch, 2012; Poppinga et al., 2012). However in 2020, *splA* gene was found in ERIC V with a frame mutation in ERIC I strains (Beims et al., 2020). Some toxins such as Plx1, Plx2 or C3larvin have been identified, but their biological function in the pathogenesis was not fully determined. The gene *plx1* have a bacteriophage origin and is present typically in ERIC I *P. larvae* genomes, through horizontal gene transfer (HGT), for other hand, *plx2* and *c3larvin*AB locus are present in all genotypes but in some of them are non-functional (Ebeling, Fünfhaus, & Genersch, 2021; Fünfhaus, Poppinga, & Genersch, 2013). For example *plx2* is interrupted in ERIC II but continue function in the other genotypes (Beims et al., 2020; Ebeling, Fünfhaus, et al., 2021; Ebeling et al., 2017; Ebeling, Pieper, et al., 2021; Fünfhaus et al., 2013), in the case of *c3larvin*AB only do not have stop mutations in ERIC III where is functional (Beims et al., 2020; Ebeling, Fünfhaus, et al., 2021; Ebeling, Knispel, Fünfhaus, & Genersch, 2019; Krska, Ravulapalli, Fieldhouse, Lugo, & Merrill, 2015; M. Turner et al., 2020).

Secondary metabolites like lantibiotics, sevadicin, paenilamicin or paenilarvin play an important role in *P. larvae* virulence not against larvae but against other bacteria or fungus that may co-exist in the larval midgut, eliminating them with these antimicrobial compounds to preserve their niche (**Figure 5**) (Djukic et al., 2014; Garcia-Gonzalez, Müller, Ensle, Süssmuth, & Genersch, 2014; Garcia-Gonzalez, Müller, Hertlein, et al., 2014; Hertlein et al., 2014; Müller et al., 2015, 2014; Schild, Fuchs, Bode, & Grünewald, 2014; Sood et al., 2014).

Nevertheless it might be assumed that the genetic variety (ERIC I-V) does not affect the capacity of *P. larvae* to infect, attack, cause the larvae death (Genersch, 2006, 2017; Genersch et al., 2005). However, each ERIC demonstrated characteristic mechanism of pathogenesis, which are not fully understood (Ebeling, Knispel, Hertlein, Fünfhaus, & Genersch, 2016; Genersch, 2017; Poppinga & Genersch, 2015).

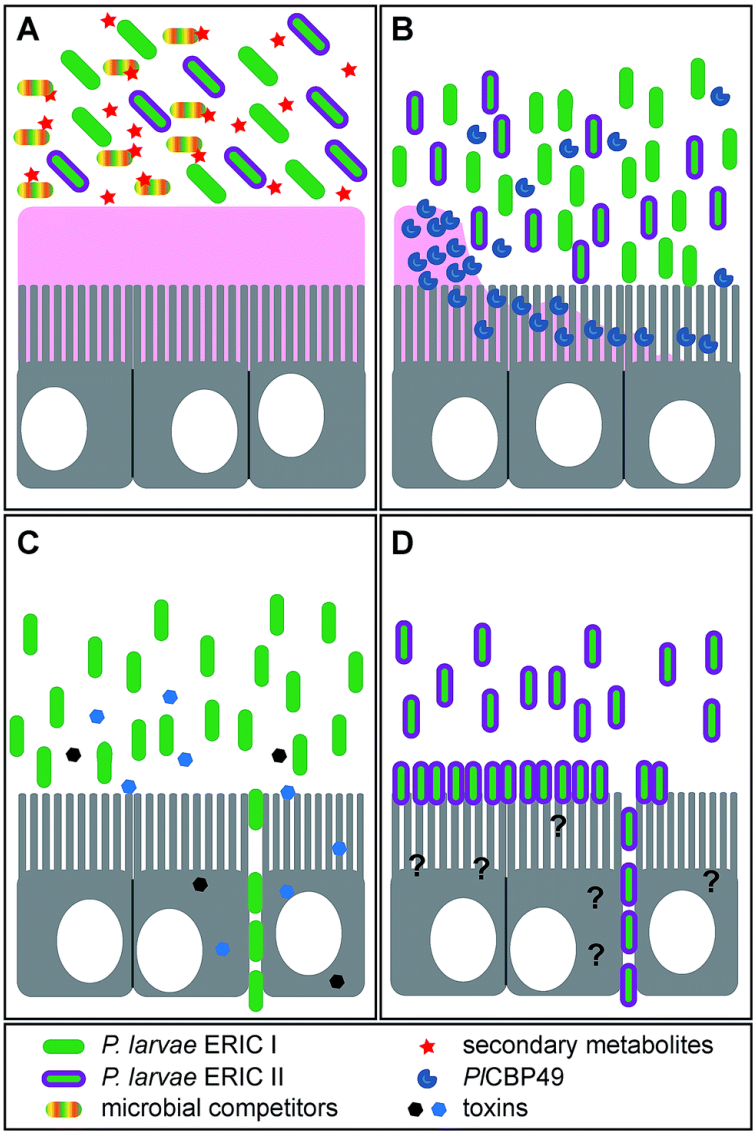

Figure 5: Role of several recently identified virulence factors during the pathogenesis of *P. larvae* infections. (A) *P. larvae* proliferating in the larval midgut will encounter microbial competitors such as other bacteria and fungus. Secondary metabolites like paenilamicin or the paenilarvins will enable *P. larvae* to defend the niche "larval gut" ensuring that a pure culture of *P. larvae* prevails in the end. (B) The larval midgut epithelium is protected by a chitin-containing peritrophic matrix (pink structure in A and B). *P. larvae* express *Pl*CBP49, a chitin-degrading enzyme, which totally degrades the peritrophic matrix. This is a key step during the pathogenesis of *P. larvae* infections, because marks the transition from the non-invasive to the invasive lifestyle of *P. larvae*. (C) Two toxins, Plx1 and Plx2, are expressed by *P. larvae* ERIC I. For both, a role as a virulence factor during pathogenesis has been experimentally demonstrated. These toxins most likely act on the unprotected epithelial cells. *P. larvae* then breach the epithelial layer via the paracellular route and invade the hemocoel. By then the infected larva is dead and *P. larvae* start to decompose the larval cadaver to a ropy mass. (D) The S-layer protein SplA is expressed by *P. larvae* ERIC II and ERIC V. SplA mediates adhesion of *P. larvae* to the midgut epithelial cells after the peritrophic matrix degradation, an important step during pathogenesis of *P. larvae* ERIC II infections. Factors used by *P. larvae* ERIC II to accomplish breaching of the epithelium and invading the hemocoel using the paracellular route via are still elusive. Adapted from Ebeling et al., 2016; Müller et al., 2015; Poppinga & Genersch, 2015.

1.5 Research aiming AFB control

Nowadays the solution used to control AFB is the burning of the complete infected hives, together with contaminated materials (brood combs, food combs, etc). This practice causes considerable economic losses to beekeeping and have great impact in ecological balance (FAO, 2006; Genersch, 2010a).

In some countries such as EUA, Canada and Japan, antibiotics are frequently used as a preventive measure (oxytetracycline (OTC), tylosin (TYL) or lincomycin (LIN)) (Forsgren, Locke, Sircoulomb, & Schäfer, 2018; Milbrath, 2021; Ueno et al., 2018). Antibiotics are non-effective against the spores of *P. larvae* and although it eliminates the vegetative form, it also harms commensal flora, beneficial to bees (Raymann & Moran, 2018). The misuse of antibiotics contributes to increase the problem of multi-resistant bacteria due to selective pressure and presently, EU regulation prohibits its use in beekeeping (EC regulation 2377/90 and further amendments (European Parliament and the Council of the European Union, 2010)). Moreover, hive derived products (honey, pollen, propolis, RJ and beeswax) contaminated with antibiotic residues are unsafe for human consumption and may hamper brood development (Genersch, 2010a; Miyagi et al., 2000; Wu, Chin, Ghalayini, & Hornitzky, 2005).

Some approaches have been presented to fight against AFB. The interference at genetic and sanitary level is in the basis of the prevention: the selection of honeybees with more skills to perform a better hygienic behaviour (Genersch, 2010a), to shook swarm into new hives when suspecting of *P. larvae* spores, to remove and burn the infected material (Milbrath, 2021), to avoid the overcrowding of colonies in the apiary respecting the distance between them, to monitor hives for early recognition of disease signs, to sterilize the handling equipment and to stop sharing frames between colonies and apiaries (Milbrath, 2021) are important measures.

However, many measures to take when the disease is being installed are being suggested and tested *in vitro* and in experimental hives. Examples of that are: the administration of commensal bacteria to brood, like *Lactobacillus* spp, *Bifidobacterium* spp and *Fructobacillus* spp, antagonist to *P. larvae* (Al-Ghamdi, Ali Khan, Javed Ansari, Almasaudi, & Al-Kahtani, 2018; Arredondo et al., 2018; Daisley et al., 2020; Forsgren, Olofsson, Vásquez, & Fries, 2009; Lamei, Stephan, Nilson, Miranda, & Forsgren, 2020; Stephan, Riesbeck, & Miranda, 2019); the use of natural antibacterial elements as essential oils of plants, relying on their hydrophobicity to promote the permeabilization and disruption of the cell and cytoplasmic membranes of *P. larvae* causing the leakage of your cytoplasmic constituents (Pellegrini, Alonso-Salces, Umpierrez, Rossini, & Fuselli, 2017; Pellegrini, Zalazar, Fuselli, & Ponce, 2017); the use of propolis or RJ, hive products with higher antimicrobial and antifungal proprieties (Borba & Spivak, 2017); the

applications of fatty acids, naturally present in larvae diet through pollen and RJ, that with its hydrophobic chain, change cell permeability resulting in cell lysis (Kuzyšinová, Mudroňová, Toporčák, Molnár, & Javorský, 2016; Laho, Koh, Mojžišov, Majt, & Klaudiny, 2018); the administration of plant extracts and bacteriocins to damage *P. larvae* cells (Alonso-Salces et al., 2017); the use of maltose reduced silver nanoparticles with short and long-term bacteriostatic and bactericidal effects against broad spectrum *Paenibacillus* sp. (Çulha et al., 2017) or inhibition of spore germination using indole- and phenol-analogue molecules with antagonistic effect to germinants (Alvarado et al., 2017). A more recent approach, planned to directly inhibit the action of a *P. larvae* toxin (Plx2A) was only successful *in vitro* (Ebeling, Pieper, et al., 2021). In other cases, the long term benefits were not proved, as happened when commensal bacteria (Daisley et al., 2020) or natural products (Alonso-Salces et al., 2017; Pellegrini, Alonso-Salces, et al., 2017) were used.

The use of bacteriophages (exclusive bacterial parasites) to control *P. larvae* in hives has been demonstrating promising results, and will be detailed bellow.

1.6 Bacteriophages (phages)

1.6.1 Generalities, advantages and lifecycle

Bacteriophages (phages), from ‘‘bacteria’’ and “phagein” (to eat or devour, in Greek), are viruses that infect and kill bacteria. They are the most abundant bio-entities present on Earth, with an estimated population of 10^{31} phages, and a huge diversity among them (Hendrix, 2003; Rohwer & Edwards, 2002). Phages are inert in extracellular environment and present in oceans, soils, air, etc. Consequently, they are consumed in foods and water, being present in human and animal microbiome (Atterbury, 2009; Kutateladze & Adamia, 2010). Phages have been studied for over 100 years. Discovered in 1915 by Frederick Twort (Twort, 1915) they were only proposed as antimicrobial agents in 1917, by Félix d’Hérelle (D’Hérelle, 1917). The firsts phage therapy trials as a therapeutic approach were promising and targeted infections such as dysentery, skin disease and cholera (Abedon, Kuhl, Blasdel, & Kutter, 2011; Sulakvelidze, Alavidze, & Morris, 2001). Due to the high number of wounded from World War II in the 1940s, the use of antibiotic like penicillin (discovered in 1928) began to be used massively by the Western world (initiating the era of antibiotics). The antibiotics were rapid, broad-spectrum and low-cost solutions (Sulakvelidze et al., 2001; Tan & Tatsumura, 2015). These characteristics combined with experimental errors in the first trials of phage therapy, (e.g.: no placebo control group, bad formulations (poor purity and stability), use of improper phages and treat non-bacterial infections with phages), led to the abandonment of phage therapy in the Western world. However, phage therapy kept being applied in countries of former Soviet Union, in Georgia and Poland (Sulakvelidze et al., 2001; Summers, Stent, & Twort, 2012). Presently, with the concern of antimicrobial resistant bacteria, interest in phage therapy has been reborn all over the globe, as multi-resistant antibiotic bacteria remain sensitive to the action of the phage (Housby & Mann, 2009; Kutateladze & Adamia, 2010; Potera, 2013).

Phages are natural bacteria predator viruses innocuous to humans, animals and plants. They exclusively and specifically recognize and infect their target hosts infecting multiplying inside and leading to their destruction (Kutateladze & Adamia, 2010). Phage specificity protects commensal microflora of the organism from the lysis. In their life cycle, phages take advantage of bacteria biosynthetic machinery, directing it toward the synthesis of more phages, which are released afterward through bacterial lysis. New phages will trigger new infection cycles in surrounding hosts, if present, resulting in an exponential growth, being though considered self-replicating antibacterial agents. Unlike antibiotics, the need of successive administration is supressed (Kutateladze & Adamia, 2010). Moreover, their isolation,

characterization and production are low-costing, which is an advantage over antibiotics (Loc-Carrillo & Abedon, 2011).

According to their lifecycle, phages can be virulent (following exclusively lytic cycle) or temperate (in a lysogenic lifestyle) (**Figure 6**). In both life cycles phages have to adsorb to host surface after recognising receptors, in order to inject the viral DNA. The lytic cycle always results in host cell lysis (action of holin–lysin system) to promote the release of a new phage progeny, while in lysogenic cycle phage can integrate the host genome (chromosome or plasmids) becoming a prophage and being able to remain at this stage for several bacterial generations. The temperate phages can, indeed, induce lysis event if external stimuli occur causing bacterial stress (L. C. Fortier & Sekulovic, 2013; Guttman, Raya, & Kutter, 2005; Hanlon, 2007; John, 2005).

Despite there are studies reporting the use of temperate phages for therapy, as for example to control *Clostridium difficile* (Meader, Mayer, Steverding, Carding, & Narbad, 2013; J. Nale, Redgwell, Millard, & Clokie, 2018; J. Y. Nale, Spencer, et al., 2016), the lytic phages are the ones indicated and safe for use in therapy. Currently, synthetic biology is being used to genetically manipulate temperate phages by removing lysogeny- and transduction-related genes or virulence-related genes (e.g.: integrases, transposases or toxins) and converting them into virulent and safer ones (Monteiro, Pires, Costa, & Azeredo, 2018; D. P. Pires, Costa, Pinto, Meneses, & Azeredo, 2020). Manipulation can also be used to improve phage performances, adding some genes with specialized function (e.g.: change the tail fibrer to increase host range (Ando, Lemire, Pires, & Lu, 2015), add some element to capsid to enhancer the phage resistance to environment (Nobrega et al., 2016) or introducing genes to improve biofilm disruption (e.g.: dispersin B) (Lu & Collins, 2007). For that, techniques such as CRISPR/Cas-mediated genome engineering or Bacteriophage Recombineering of Electroporated DNA (BRED) are being explored (Costa, Milho, Azeredo, & Pires, 2018; D. P. Pires, Cleto, Sillankorva, Azeredo, & Lu, 2016; D. P. Pires et al., 2021).

Nowadays, phages are already being applied in agriculture (Adriaenssens et al., 2012; Y. J. Bae et al., 2012), food safety (Bigwood, Hudson, Billington, Carey-Smith, & Heinemann, 2008; Leverentz et al., 2003; Spricigo, Bardina, Cortés, & Llagostera, 2013) or veterinary medicine (C. Carvalho, Costa, Silva, & Oliveira, 2017; C. M. Carvalho et al., 2010; Hawkins, Harper, Burch, Änggård, & Soothill, 2010; Oliveira, Sereno, & Azeredo, 2010) and commercial phages products are already available.

The taxonomic division of phages is from the responsibility of the International Committee on Taxonomy of Viruses (ICTV), which are constantly improving with rigorous criteria the classification (Adriaenssens & Rodney Brister, 2017; Adriaenssens et al., 2020). For several years the phages were

grouped in the three main families the *Myoviridae*, the *Siphoviridae* and the *Podoviridae* of the *Caudovirales* order, based on their tail morphology and type of nucleic acid, however, the abolishment these classifications were already proposed (D. Turner, Kropinski, & Adriaenssens, 2021), and now were defined 14 families (https://talk.ictvonline.org/taxonomy/p/taxonomy_releases accessed on 30 November 2021) (Adriaenssens et al., 2020).

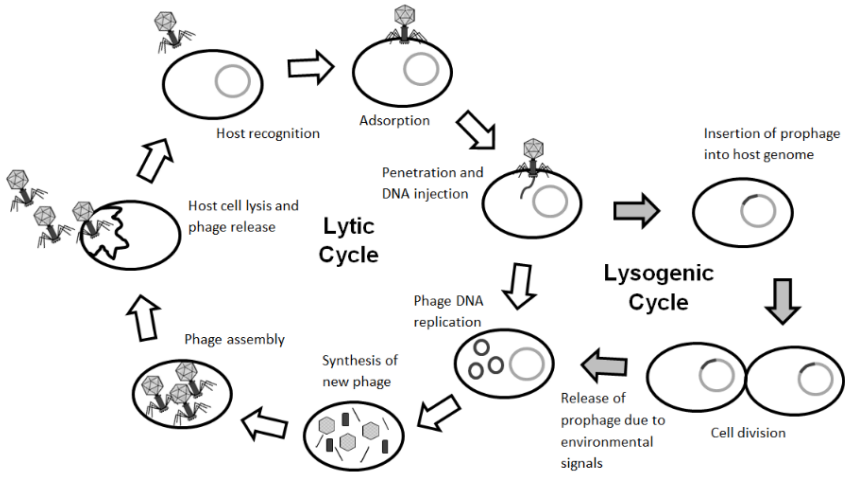

Figure 6: The phage life cycles. Virulent phage only can go through the lytic cycle, resulting in the host lysis to promote the progeny release of new phages. Temperate phages can decide between the lytic or the lysogenic cycle. In the lysogenic life cycle phage can insert their genome into the host bacterial genome (chromosome or plasmids), when it is insert is known as prophage, and can be induced by external stress factors excising it, switching to lytic cycle. Adapted from Doss, Culbertson, Hahn, Camacho, & Barekzi, 2017.

1.6.2 Exploring *P. larvae* phages and their genes

The first time *P. larvae* phages were reported was in the 1950s by Smirnova, (1953) and Gochnauer, (1955) and until 1999, nine more phages were isolated: the BLA (Drobníková & Ludvík, 1982), L3 (Popova, Valerianov, & Toschkoff, 1976), BL2 (Benada, Ludvík, & Drobníková, 1984), PBL1 and PBL1c (Dingman, Bakhiet, Field, & Stahly, 1984), PBL0.5 (Bakhiet & Stahly, 1988), PBL2 (Bakhiet & Stahly, 1988) PBL3 (Campana, Bakhiet, & Stahly, 1991) and PPL1c (Stahly et al., 1999). However, only in 2013, the first complete *P. larvae* phage genome, phiIBB_Pl23 (KF010834), was deposited on GenBank (Oliveira, Melo, Kropinski, & Azeredo, 2013). Since then, other full *P. larvae* phage genomes were published (**Table 1**). So far 51 *P. larvae* phages were characterized, sequenced, and the genome and proteome were analysed. Most of them (n=49) belongs to the *Siphoviridae* family (Beims et al., 2020; A. Oliveira et al., 2013; Stamereilers et al., 2018) and only two were recently classified as *Podoviridae* (vB_PlaP_API480 (Ribeiro, Melo, et al., 2019) and vB_PlaP_SV21 (Bozdeveci, Akpınar, & Karaoğlu, 2021)). All these phages seem to be temperate, following a lysogenic lifecycle.

Studies performed out to date allowed to group all *P. larvae* phages with the name of the most representative ones, based on whole-genome nucleotide sequence identity: five clusters, Fern, Harrison, Vegas, Halcyone and API480 and a singleton, Lily (Bozdeveci et al., 2021; Ribeiro, Melo, et al., 2019; Stamereilers et al., 2018). In general, structural and assembly genes are conserved at the beginning of the phages genomes from the several clusters, contrarily to replication and regulatory genes, in the middle and final regions of genome, which are not conserved even within the same cluster.

Despite this diversity, most of these phages encode for example the large terminase, one or more transposases, the HicA toxin and HicB antitoxin (Stamereilers et al., 2018), and all encode a N-acetylmuramoyl-L-alanine amidase, the most conserved protein that serves as an endolysin (Stamereilers et al., 2018). Endolysins from a Gram-positive background are modular, displaying an enzymatic or catalytic active domain (EAD) at the N-terminal and also a cell binding domain (CBD) at C-terminal section. The EAD is responsible to cleave the amide bonds between the α-amino group of L-alanine and the lactyl group of the muramic acid of the peptidoglycan resulting in the loss of the integrity of the host cells. The CBD control enzyme specificity and allows recognition of the host. For having such functions, endolysins are the only proteins whose function was experimentally validated in the *P. larvae* phage population and were studied envisaging either therapeutical applications for AFB (LeBlanc et al., 2015; A. Oliveira et al., 2015) or *P. larvae* detection (through CBD) (Santos, Oliveira, Melo, & Azeredo, 2019). The next section will detail assays involving phages and their endolysins.

Table 1: *P. larvae* phages sequenced and available on NCBI. Phages are grouped by the date of the genome announcement and the GenBank Accession Number.

Date	Phage	GenBank Accession No.	Reference
2013	phiIBB_PI23	KF010834	(Oliveira, Melo, Kropinski, & Azeredo, 2013)
2015	HB10c2	KP202972	(Beims et al., 2015)
	Diva	KP296791	(Carson et al., 2015)
	Lily	KP296792	
	Rani	KP296793	
	Redbud	KP296794	
	Shelly	KP296795	
	Sitara	KP296796	
	Fern	KT361649	(Tsourkas et al., 2015)
	Willow	KT361650	
	Harrison	KT361651	
	Xenia	KT361652	
	Paisley	KT361653	

	Vegas	KT361654	
	Hayley	KT361655	
	Vadim	KT361656	
	Diane	KT361657	
2016	Tripp	KT755656	(Abraham et al., 2016)
2018	BN12	MG727695	(Walker et al., 2018)
	Kiel007	MG727696	
	Dragolir	MG727697	
	PBL1c [1]	MG727698	
	Pagassa	MG727699	
	Tadhana	MG727700	
	Leyra	MG727701	
	Likha	MG727702	
	Wanderer	MH431930	(B.D Merrill et al., 2018)
	Yerffej	MH431931	
	Arcticfreeze	MH431932	
	DevRi	MH431933	
	Gryphonian	MH431934	
	Honeybear	MH431935	
	Kawika	MH431936	
	Lucielle	MH431937	
	C7Cdelta	MH431938	
	Ash	MH454076	
	Bloom	MH454077	
	Eltigre	MH454078	
	Jacopo	MH454079	
	Ley	MH454080	
	LincolnB	MH454081	
	Genki	MH454082	
	Saudage	MH454083	
	Toothless	MH454084	
	Unity	MH460824	(Yost et al., 2018)
	Scottie	MH460825	
	Heath	MH460826	
	Halcyone	MH460827	
2019	vB_PlaP_API480	MK533143.1	(Ribeiro, Melo, et al., 2019)
2020	phiERICV	CP019719.1	(Beims et al., 2020)

2021	vB_PlaP_SV21	MZ218124	(Bozdeveci et al., 2021)

(1) – PBL1c original isolated in 1984 (Dingman et al., 1984) but sequenced in 2018 (Walker et al., 2018).

1.6.3 Experiments involving *P. larvae* phages and endolysins

Since 2015, efforts have been made to explore the potential of *P. larvae* phages (Beims et al., 2015; Brady et al., 2017; Ghorbani-Nezami et al., 2015; Yost et al., 2016) or/and respective endolysins (LeBlanc et al., 2015) as a tool for treating AFB (**Table 2**). Three laboratory-raised larvae studies (Beims et al., 2015; Ghorbani-Nezami et al., 2015; Yost et al., 2016), showed that, phages were not toxic to larvae. The prophylactic treatment with phages, administered to larvae 24 hours before challenge with *P. larvae* spores, revealed that two out of three tested phages allowed to equal the larval survival rate of the negative control group (Ghorbani-Nezami et al., 2015). Similarly, Beims et al., (2015) concluded that prophylaxis can be an useful approach. Moreover, they reported that phages in cocktails may be more effective than individually. Yost et al., (2016), using phages in a cocktail, reported an increase in the larvae survival after exposure to *P. larvae* spores, either when used prophylactically or as a treatment.

The first experiment performed in hive conditions was performed by Brady and colleagues (2017), who evaluated the effectiveness of a phage cocktail in decreasing larvae mortality, after spraying (5×10^{8} $PFU.mL^{-1}$) over larvae combs. They could observe that phages a not only prevented the AFB infection (100% efficient for the at-risk hives), but also efficiently treated the infected hives (100% of the hives still recoverable were recovered in six weeks). Furthermore, they proved that an overdose of phage is safe for the hive, reporting that antibiotic treatment causes more hive losses than the treatment with phage (Brady et al., 2017).

Later, in 2019, a study investigated the efficacy of phage oral administration to honeybees, in providing phages to larvae, where phage bioavailability in larvae tissues were evaluated (Ribeiro, Correia, et al., 2019). This pioneer study demonstrated that phages were able to reach of bees and larvae tissues, but in different concentrations and viability state. While, in average 10^{4} phages per larvae was detected in larvae tissues by quantitative PCR, only an average of 32 phages per larvae were available in an infectious state. However, phages were present in the midgut of larvae, the site of *P. larvae* infection, in enough amount to protect the larvae from AFB and thus, it suggests the need of protecting phages from the harsh environment of the hive (low pH, enzymes, RJ, phenolic compounds, etc.) to increase the efficacy of the treatment (Ribeiro, Correia, et al., 2019).

All these studies pointed out that an effective treatment of AFB largely depends on the *P. larvae* phages selected, their spectra lytic and their specificity, avoiding commensal damage (did not disturbed gut microbiome), the methods of administration to larvae and the virulence of the *P. larvae* strains.

Concerning endolysins, their potential use for controlling AFB was approached in two works. The first, in 2015, characterized the endolysin PlyPl23 from phage phiIBB_Pl23 (A. Oliveira et al., 2015), and, in the same year, another reported PlyPalA from Xenia phage (LeBlanc et al., 2015). Both endolysins PlyPl23 and PlyPalA were described *in vitro* and using laboratory-reared larvae. Their optimal temperature of action is 35 °C like inside of the hives, both improved their activity with ionic strength (200 mM and 150 mM respectively). They did not reveal any sporicidal activity or perturbation in the commensal flora and tolerate lyophilization without loss of activity. The optimal pH (pH=7) of PlyPl23 is similar to the existent inside of larval guts, do not demonstrate toxic effect to larvae and has a synergic antimicrobial power when mixed with RJ. The assays with 0.2 µM PlyPl23 against *P. larvae* (10^5 CFU.mL^{-1}) performed 1 hour after of incubation revealed a 3.3 CFU.mL^{-1} Log reduction (enough to control *P. larvae* in larvae). PlyPalA, has the optimal pH (pH=8), display 50% of activity at the pH of larval gut, has a zinc ligation site and lose their activity with chelator EDTA. When administered 100 µg/mL PlyPalA at *P. larvae* suspension (10^5 CFU.mL^{-1}) a reduction of 1.8 Log CFU.mL^{-1}, was observed. However, a single dose of PlyPalA (16 µg/mL) rescued 75% of larvae infected with spores, showing that it represents a powerful tool for future treatment of AFB.

To date, no bacterial resistance to endolysins has been reported and how it can be applied externally in Gram-positive bacteria without the presence of holins, because the peptidoglycan is exposed and accessible to its action (Murray, Draper, Ross, & Hill, 2021).

Further studies need to be designed to achieve the best formulations of phages and endolysins (lytic spectra, cocktails, concentrations, application routes, encapsulations formulas, adjuvants, etc.). Field experiments in healthy and contaminated hives should be performed, to understand the prophylactic and treatment effect and to estimate the ideal duration of the treatment considering that spores do not germinate all at the same time and evaluating the defence effects from bees, such as hygienic behaviour.

Table 2: Application of phages or endolysin in bees. Adapted from Jończyk-Matysiak et al., (2020).

Applied phages or endolysin	Mode of treatment	Results and recommendations	References
HB10c2 *P. larvae* phage	Lab-reared larvae infected with spores at 500 CFU/larva and phages applied at a concentration of 50000 PFU/larva	Phage did not cause larvae mortality and did not disturb gut microbiota composition. However, phage therapy was not efficient in AFB treatment in infected larvae	(Beims et al., 2015)
F, WA, XIII *P. larvae* phages	Lab-reared larvae infected with 1000 spores each and phages applied at a concentration of 10^5–10^7 PFU/mL individually or as a cocktail in day 0 or day 1	Phage did not cause larvae mortality. Phages applied as prophylactic measures decreased larval mortality.	(Ghorbani-Nezami et al., 2015)
Xenia, Halcyone, Willow, Fern, Vadim, Harrison and Hayley *P. larvae* phages	Lab-reared larvae infected with increasing amounts of spores for 1 weak. Cocktail phages applied at a concentration of 10^6 PFU/mL before and after 4 hours of spores	Phage did not cause larvae mortality. Experiments indicated that administration of a phage cocktail applied as prophylactic, or treatment resulted in a 59% survival increase of larvae.	(Yost et al., 2016)
PlyPl23 lysin from *P. larvae* phage phiIBB_Pl23	Assessment of toxicity in lab-reared larvae with 0.2 μM and *in vitro* evaluation and characterization.	Endolysin did not cause larvae mortality and did not disturb gut microbiota composition. Evidence a synergic antimicrobial power when mixed with RJ. Revealed a 3.3 $CFU.mL^{-1}$ Log reduction of *P. larvae* suspension (10^5 $CFU.mL^{-1}$) with 0.2 μM (1 hour).	(A. Oliveira et al., 2015)
PlyPalA lysin from *P. larvae* phage Xenia	Lab-reared larvae infected with 1000 spores each and endolysin applied at a concentration of 16 μg/mL and *in vitro* evaluation and characterization.	Endolysin did not cause larvae mortality and did not disturb gut microbiota composition. A single dose of the endolysin were rescued 75% of the infected larvae.	(LeBlanc et al., 2015)
1, 5, 9 *P. larvae* phages isolated here	Hive infected with signs of AFB and healthy hives. Cocktail phages sprayed in the combs with a concentration of 10^6 PFU/mL by three times a day for 10 days.	Phage did not cause deaths (mortality) in health hives and did not disturb gut microbiota composition even after an overdose application. Phages safer comparatively to antibiotic treatment.	(Brady et al., 2017)

		Protective and therapeutic effects were observed in this study	
T7 *Escherichia coli* phage	Healthy hives administrated with a single dose of phage for 24 hours. Phage concentration of 10^9 PFU/mL applied by oral route.	Phage did not cause deaths (mortality) in health hives and did not disturb gut microbiota. The oral catch route for bees allowed detect viable phages in larvae (few phages). Hives environments is harsh for phage viability, royal jelly damage all phage in 3 hours. Phages needs some type of protection or encapsulation.	(Ribeiro, Correia, et al., 2019)

References

Abedon, S. T., Kuhl, S. J., Blasdel, B. G., & Kutter, E. M. (2011). Phage treatment of human infections. *Bacteriophage*, *1*(2), 66–85. https://doi.org/10.4161/bact.1.2.15845

Abraham, J., Bousquet, A., Bruff, E., Carson, N., Clark, A., Connell, A., ... Miller, E. S. (2016). Paenibacillus larvae Phage Tripp Genome Has 378-Base-Pair Terminal Repeats. *Genome Announcements*, *4*(1), 1498–15. https://doi.org/10.1128/genomeA.01498-15

Adriaenssens, E. M., & Rodney Brister, J. (2017). How to name and classify your phage: An informal guide. *Viruses*, *9*(4), 1–9. https://doi.org/10.3390/v9040070

Adriaenssens, E. M., Sullivan, M. B., Knezevic, P., van Zyl, L. J., Sarkar, B. L., Dutilh, B. E., ... Krupovic, M. (2020). Taxonomy of prokaryotic viruses: 2018-2019 update from the ICTV Bacterial and Archaeal Viruses Subcommittee. *Archives of Virology*. https://doi.org/10.1007/s00705-020-04577-8

Adriaenssens, E. M., Van Vaerenbergh, J., Vandenheuvel, D., Dunon, V., Ceyssens, P.-J., De Proft, M., ... Lavigne, R. (2012). T4-Related Bacteriophage LIMEstone Isolates for the Control of Soft Rot on Potato Caused by 'Dickeya solani.' *PLoS ONE*, *7*(3), e33227. https://doi.org/10.1371/journal.pone.0033227

Al-Ghamdi, A., Ali Khan, K., Javed Ansari, M., Almasaudi, S. B., & Al-Kahtani, S. (2018). Effect of gut bacterial isolates from Apis mellifera jemenitica on Paenibacillus larvae infected bee larvae. *Saudi Journal of Biological Sciences*, *25*(2), 383–387. https://doi.org/10.1016/j.sjbs.2017.07.005

Alonso-Salces, R. M., Cugnata, N. M., Guaspari, E., Pellegrini, M. C., Aubone, I., De Piano, F. G., ... Fuselli, S. R. (2017). Natural strategies for the control of Paenibacillus larvae, the causative agent of American foulbrood in honey bees: a review. *Apidologie*, *48*(3), 387–400. https://doi.org/10.1007/s13592-016-0483-1

Alvarado, I., Margotta, J. W., Aoki, M. M., Flores, F., Agudelo, F., Michel, G., ... Abel-Santos, E. (2017). Inhibitory effect of indole analogs against Paenibacillus larvae, the causal agent of American foulbrood disease. *Journal of Insect Science*, *17*(5). https://doi.org/10.1093/jisesa/iex080

Alvarado, I., Phui, A., Elekonich, M. M., & Abel-Santos, E. (2013). Requirements for in vitro germination of Paenibacillus larvae spores. *Journal of Bacteriology*, *195*(5), 1005–1011. https://doi.org/10.1128/JB.01958-12

Ando, H., Lemire, S., Pires, D. P., & Lu, T. K. (2015). Engineering Modular Viral Scaffolds for Targeted Bacterial Population Editing. *Cell Systems*, *1*(3), 187–196. https://doi.org/10.1016/j.cels.2015.08.013

Antúnez, K., Piccini, C., Castro-Sowinski, S., Rosado, A. S., Seldin, L., & Zunino, P. (2007). Phenotypic and genotypic characterization of Paenibacillus larvae isolates. *Veterinary Microbiology*, *124*(1–2), 178–183. https://doi.org/10.1016/j.vetmic.2007.04.012

Arredondo, D., Castelli, L., Porrini, M. P., Garrido, P. M., Eguaras, M. J., Zunino, P., & Antúnez, K. (2018). Lactobacillus kunkeei strains decreased the infection by honey bee pathogens Paenibacillus larvae and Nosema ceranae. *Beneficial Microbes*, *9*(2), 279–290. https://doi.org/10.3920/BM2017.0075

Ashiralieva, A., & Genersch, E. (2006). Reclassification, genotypes and virulence of Paenibacillus larvae , the etiological agent of American foulbrood in honeybees – a review. *Apidologie*, *37*(4), 411–420. https://doi.org/10.1051/apido:2006028

Atterbury, R. J. (2009). Bacteriophage biocontrol in animals and meat products, *2*, 601–612. https://doi.org/10.1111/j.1751-7915.2009.00089.x

Bae, Y. J., Wu, J., Lee, H. J., Jo, E. J., Murugaiyan, S., Chung, E., & Lee, S. W. (2012). Biocontrol potential of a lytic bacteriophage PE204 against bacterial wilt of tomato. *Journal of Microbiology and Biotechnology*, *22*(12), 1613–1620. https://doi.org/10.4014/jmb.1208.08072

Bakhiet, N., & Stahly, D. P. (1988). Properties of clear plaque mutants of the Bacillus larvae bacteriophages PBL0.5 and PBL2. *Journal of Invertebrate Pathology*, *52*(1), 78–83. https://doi.org/10.1016/0022-2011(88)90105-X

Barnutiu, L. I., Marghitas, L. Al, Dezmirean, D. S., Mihai, C. M., & Bobis, O. (2011). Chemical composition and antimicrobial activity of royal jelly - Review. *Animal Science and Biotechnologies*, *44*(2), 67–72.

Bassi, S., Formato, G., Milito, M., Trevisiol, K., Salogni, C., & Carra, E. (2015). Phenotypic characterization and ERIC–PCR based genotyping of Paenibacillus larvae isolates recovered from American foulbrood outbreaks in honey bees from Italy. *Veterinary Quarterly*, *35*(1), 27–32. https://doi.org/10.1080/01652176.2014.993095

Beims, H., Bunk, B., Erler, S., Mohr, K. I., Spröer, C., Pradella, S., ... Steinert, M. (2020). Discovery of Paenibacillus larvae ERIC V: Phenotypic and genomic comparison to genotypes ERIC I-IV reveal different inventories of virulence factors which correlate with epidemiological prevalences of American Foulbrood. *International Journal of Medical Microbiology*, *310*(2), 151394. https://doi.org/10.1016/j.ijmm.2020.151394

Beims, H., Wittmann, J., Bunk, B., Spröer, C., Rohde, C., Günther, G., ... Steinert, M. (2015). Paenibacillus larvae-Directed bacteriophage HB10c2 and its application in American foulbrood-affected honey bee larvae. *Applied and Environmental Microbiology*, *81*(16), 5411–5419. https://doi.org/10.1128/AEM.00804-15

Benada, O., Ludvík, J., & Drobníková, V. (1984). Morphology of a new bacteriophage isolated from Bacillus larvae. *Folia Microbiologica*, *29*(6), 520–521. https://doi.org/10.1007/BF02873162

Bigwood, T., Hudson, J. A., Billington, C., Carey-Smith, G. V., & Heinemann, J. A. (2008). Phage inactivation of foodborne pathogens on cooked and raw meat. *Food Microbiology*, *25*(2), 400–406. https://doi.org/10.1016/j.fm.2007.11.003

Biová, J., Bzdil, J., Dostálková, S., Petřivalský, M., Brus, J., Carra, E., & Danihlík, J. (2021). American Foulbrood in the Czech Republic: ERIC II Genotype of Paenibacillus Larvae Is Prevalent. *Frontiers in Veterinary Science*, *8*(August), 1–5. https://doi.org/10.3389/fvets.2021.698976

Borba, R. S., & Spivak, M. (2017). Propolis envelope in Apis mellifera colonies supports honey bees against the pathogen, Paenibacillus larvae. *Scientific Reports*, *7*(1), 11429. https://doi.org/10.1038/s41598-017-11689-w

Bozdeveci, A., Akpınar, R., & Karaoğlu, Ş. A. (2021). Isolation, characterization, and comparative genomic analysis of vB_PlaP_SV21, new bacteriophage of Paenibacillus larvae. *Virus Research*, *305*(September), 198571. https://doi.org/10.1016/j.virusres.2021.198571

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of Paenibacillus larvae in honeybee hives. *Journal of Invertebrate Pathology*, *150*(September), 94–100. https://doi.org/10.1016/j.jip.2017.09.010

Campana, C. F., Bakhiet, N., & Stahly, D. P. (1991). Morphology of Bacillus larvae bacteriophage PBL3 and physical map of its DNA. *Journal of Invertebrate Pathology*, *57*(1), 141–143. https://doi.org/10.1016/0022-2011(91)90055-U

Carreck, N. L., Andree, M., Brent, C. S., Cox-foster, D., Dade, H. A., Ellis, J. D., & Hatjina, F. (2013). Standard methods for Apis mellifera anatomy and dissection. *Journal of Apicultural Research*, *52*(4), 1–40. https://doi.org/10.3896/IBRA.1.52.4.03

Carson, S., Bruff, E., DeFoor, W., Dums, J., Groth, A., Hatfield, T., … Miller, E. S. (2015). Genome Sequences of Six Paenibacillus larvae Siphoviridae Phages. *Genome Announcements*, *3*(3), e00101-15. https://doi.org/10.1128/genomeA.00101-15

Carvalho, C., Costa, A. R., Silva, F., & Oliveira, A. (2017). Bacteriophages and their derivatives for the treatment and control of food-producing animal infections. *Critical Reviews in Microbiology*, *43*(5), 583–601. https://doi.org/10.1080/1040841X.2016.1271309

Carvalho, C. M., Gannon, B. W., Halfhide, D. E., Santos, S. B., Hayes, C. M., Roe, J. M., & Azeredo, J. (2010). The in vivo efficacy of two administration routes of a phage cocktail to reduce numbers of Campylobacter coli and Campylobacter jejuni in chickens. *BMC Microbiology*, *10*. https://doi.org/10.1186/1471-2180-10-232

Chan, Q. W. T., Yi Chan, M., Logan, M., Fang, Y., Higo, H., & Foster, L. J. (2013). Honey bee protein atlas at organ-level resolution. *Genome Research*, *23*(11), 1951–1960. https://doi.org/10.1101/gr.155994.113

Chen, C. (1995). Changes in protein components and storage stability of royal jelly under various conditions. *Food Chemistry*, *54*(2), 195–200. https://doi.org/10.1016/0308-8146(95)00031-D

Colibar, O., Popovici, D., Eugeniu, C., & Korodi, G. (2010). The effect of acidifiant on the development of bee families (Apis mellifica). *Med. Vet.*, *43*(2), 296–299.

Corby-Harris, V., Deeter, M. E., Snyder, L., Meador, C., Welchert, A. C., Hoffman, A., & Obernesser, B. T. (2020). Octopamine mobilizes lipids from honey bee (Apis mellifera) hypopharyngeal glands. *The Journal of Experimental Biology*, *223*(8), jeb216135. https://doi.org/10.1242/jeb.216135

Costa, A. R., Milho, C., Azeredo, J., & Pires, D. P. (2018). Bacteriophage Therapy, *1693*, 285–300. https://doi.org/10.1007/978-1-4939-7395-8

Crailsheim, K. (1990). The protein balance of the honey bee worker. *Apidologie*, *21*(5), 417–429. https://doi.org/10.1051/apido:19900504

Crailsheim, Karl. (1992). The flow of jelly within a honeybee colony. *Journal of Comparative Physiology B*, *162*(8), 681–689. https://doi.org/10.1007/BF00301617

Crailsheim, Karl, & Riessberger-Gallé, U. (2001). Honey bee age-dependent resistance against American foulbrood. *Apidologie*, *32*(1), 91–103. https://doi.org/10.1051/apido:2001114

Çulha, M., Kalay, Ş., Sevim, E., Pinarbaş, M., Baş, Y., Akpinar, R., & Karaoğlu, Ş. A. (2017). Biocidal properties of maltose reduced silver nanoparticles against American foulbrood diseases pathogens. *BioMetals*, *30*(6), 893–902. https://doi.org/10.1007/s10534-017-0055-5

D'Hérelle, F. (1917). Sur un microbe invisible antagoniste des bacilles dysentériques. *Research in Microbiology*, *7081*. https://doi.org/10.4161/bact.1.1.14941

Daisley, B. A., Pitek, A. P., Chmiel, J. A., Al, K. F., Chernyshova, A. M., Faragalla, K. M., … Reid, G. (2020). Novel probiotic approach to counter Paenibacillus larvae infection in honey bees. *The ISME Journal*, *14*(2), 476–491. https://doi.org/10.1038/s41396-019-0541-6

Deboutte, W., Beller, L., Yinda, C. K., Maes, P., de Graaf, D. C., & Matthijnssens, J. (2020). Honey-bee–associated prokaryotic viral communities reveal wide viral diversity and a profound metabolic coding potential. *Proceedings of the National Academy of Sciences*, 201921859. https://doi.org/10.1073/pnas.1921859117

DeGrandi-Hoffman, G., & Hagler, J. (2000). The flow of incoming nectar through a honey bee (Apis mellifera L.) colony as revealed by a protein marker. *Insectes Sociaux*, *47*(4), 302–306. https://doi.org/10.1007/PL00001720

Dingman, D. W., Bakhiet, N., Field, C. C., & Stahly, D. P. (1984). Isolation of two bacteriophages from Bacillus larvae, PBL1

and PBL0.5, and partial characterization of PBL1. *Journal of General Virology*, *65*, 1101–1105.
Djukic, M., Brzuszkiewicz, E., Fünfhaus, A., Voss, J., Gollnow, K., Poppinga, L., ... Daniel, R. (2014). How to kill the honey bee larva: Genomic potential and virulence mechanisms of Paenibacillus larvae. *PLoS ONE*, *9*(3), e90914. https://doi.org/10.1371/journal.pone.0090914
Doss, J., Culbertson, K., Hahn, D., Camacho, J., & Barekzi, N. (2017). A Review of Phage Therapy against Bacterial Pathogens of Aquatic and Terrestrial Organisms. *Viruses*, *9*(3), 50. https://doi.org/10.3390/v9030050
Drobníková, V., & Ludvík, J. (1982). Bacteriophage of Bacillus Larvae. *Journal of Apicultural Research*, *21*(1), 53–56. https://doi.org/10.1080/00218839.1982.11100516
Ebeling, J., Fünfhaus, A., & Genersch, E. (2021). The Buzz about ADP-Ribosylation Toxins from Paenibacillus larvae, the Causative Agent of American Foulbrood in Honey Bees. *Toxins*, *13*(2), 151. https://doi.org/10.3390/toxins13020151
Ebeling, J., Fünfhaus, A., Knispel, H., Krska, D., Ravulapalli, R., Heney, K. A., ... Genersch, E. (2017). Characterization of the toxin Plx2A, a RhoA-targeting ADP-ribosyltransferase produced by the honey bee pathogen Paenibacillus larvae. *Environmental Microbiology*, *19*(12), 5100–5116. https://doi.org/10.1111/1462-2920.13989
Ebeling, J., Knispel, H., Fünfhaus, A., & Genersch, E. (2019). The biological role of the enigmatic C3larvinAB toxin of the honey bee pathogenic bacterium Paenibacillus larvae. *Environmental Microbiology*, *21*, 3091–3106. https://doi.org/10.1111/1462-2920.14709
Ebeling, J., Knispel, H., Hertlein, G., Fünfhaus, A., & Genersch, E. (2016). Biology of Paenibacillus larvae, a deadly pathogen of honey bee larvae. *Applied Microbiology and Biotechnology*, *100*(17), 7387–7395. https://doi.org/10.1007/s00253-016-7716-0
Ebeling, J., Pieper, F., Göbel, J., Knispel, H., McCarthy, M., Goncalves, M., ... Genersch, E. (2021). Anti-Virulence Strategy against the Honey Bee Pathogenic Bacterium Paenibacillus larvae via Small Molecule Inhibitors of the Bacterial Toxin Plx2A. *Toxins*, *13*(9), 607. https://doi.org/10.3390/toxins13090607
Erban, T., Zitek, J., Bodrinova, M., Talacko, P., Bartos, M., & Hrabak, J. (2019). Comprehensive proteomic analysis of exoproteins expressed by ERIC I, II, III and IV Paenibacillus larvae genotypes reveals a wide range of virulence factors. *Virulence*, *10*(1), 363–375. https://doi.org/10.1080/21505594.2019.1603133
European Commission. (2019). Report from the commission to the European Parliament and the council. On the implementation of apiculture programmes, *53*(9), 1689–1699.
European Commission. (2021). Honey Market Presentation - Spring 2021, 23. Retrieved from https://ec.europa.eu/info/sites/default/files/food-farming-fisheries/animals_and_animal_products/documents/market-presentation-honey-spring2021_en.pdf
European Parliament and the Council of the European Union. (2010). Commission regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuff of animal origin. *Official Journal of the European Union*, (L 15/1).
Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J. L., Jiang, H., ... Hultmark, D. (2006). Immune pathways and defence mechanisms in honey bees Apis mellifera. *Insect Molecular Biology*, *15*(5), 645–656. https://doi.org/10.1111/j.1365-2583.2006.00682.x
FAO. (2006). Honey bee diseases and pests: a practical guide. *Agricultural and Food Engineering Technical Report*, (4), 27.
Faux, C. M. (2021). Honey Bee Anatomy. *Honey Bee Medicine for the Veterinary Practitioner*, 33–40. https://doi.org/10.1002/9781119583417.ch3
Forsgren, E., Locke, B., Sircoulomb, F., & Schäfer, M. O. (2018). Bacterial Diseases in Honeybees. *Current Clinical Microbiology Reports*, *5*, 18. https://doi.org/10.1007/s40588-018-0083-0
Forsgren, E., Olofsson, T. C., Vásquez, A., & Fries, I. (2009). Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honey bee larvae. *Apidologie*, *41*(1), 99–108. https://doi.org/10.1051/apido/2009065
Forsgren, E., Stevanovic, J., & Fries, I. (2008). Variability in germination and in temperature and storage resistance among Paenibacillus larvae genotypes. *Veterinary Microbiology*, *129*(3–4), 342–349. https://doi.org/10.1016/j.vetmic.2007.12.001
Fortier, L. C., & Sekulovic, O. (2013). Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence*, *4*(5), 354–365. https://doi.org/10.4161/viru.24498
Fünfhaus, A., & Genersch, E. (2012). Proteome analysis of Paenibacillus larvae reveals the existence of a putative S-layer protein. *Environmental Microbiology Reports*, *4*(2), 194–202. https://doi.org/10.1111/j.1758-2229.2011.00320.x
Fünfhaus, A., Poppinga, L., & Genersch, E. (2013). Identification and characterization of two novel toxins expressed by the lethal honey bee pathogen P aenibacillus larvae, the causative agent of American foulbrood. *Environmental Microbiology*, *15*(11), n/a-n/a. https://doi.org/10.1111/1462-2920.12229
Garcia-Gonzalez, E., & Genersch, E. (2013). Honey bee larval peritrophic matrix degradation during infection with Paenibacillus larvae, the aetiological agent of American foulbrood of honey bees, is a key step in pathogenesis. *Environmental Microbiology*, *15*(11), 2894–2901. https://doi.org/10.1111/1462-2920.12167
Garcia-Gonzalez, E., Müller, S., Ensle, P., Süssmuth, R. D., & Genersch, E. (2014). Elucidation of sevadicin, a novel non-ribosomal peptide secondary metabolite produced by the honey bee pathogenic bacterium P aenibacillus larvae.

Environmental Microbiology, *16*(5), 1297–1309. https://doi.org/10.1111/1462-2920.12417

Garcia-Gonzalez, E., Müller, S., Hertlein, G., Heid, N., Süssmuth, R. D., & Genersch, E. (2014). Biological effects of paenilamicin, a secondary metabolite antibiotic produced by the honey bee pathogenic bacterium Paenibacillus larvae. *MicrobiologyOpen*, *3*(5), 642–656. https://doi.org/10.1002/mbo3.195

Garcia-Gonzalez, E., Poppinga, L., Fünfhaus, A., Hertlein, G., Hedtke, K., Jakubowska, A., & Genersch, E. (2014). Paenibacillus larvae Chitin-Degrading Protein PlCBP49 Is a Key Virulence Factor in American Foulbrood of Honey Bees. *PLoS Pathogens*, *10*(7), e1004284. https://doi.org/10.1371/journal.ppat.1004284

Genersch, E. (2006). Reclassification of Paenibacillus larvae subsp. pulvifaciens and Paenibacillus larvae subsp. larvae as Paenibacillus larvae without subspecies differentiation. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, *56*(3), 501–511. https://doi.org/10.1099/ijs.0.63928-0

Genersch, E. (2010a). American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. *Journal of Invertebrate Pathology*, *103*(SUPPL. 1), S10–S19. https://doi.org/10.1016/j.jip.2009.06.015

Genersch, E. (2010b). Honey bee pathology: current threats to honey bees and beekeeping. *Applied Microbiology and Biotechnology*, *87*(1), 87–97. https://doi.org/10.1007/s00253-010-2573-8

Genersch, E. (2017). Beekeeping – From Science to Practice, 157–174. https://doi.org/10.1007/978-3-319-60637-8

Genersch, E., Ashiralieva, A., & Fries, I. (2005). Strain- and Genotype-Specific Differences in Virulence of Paenibacillus larvae subsp. larvae, a Bacterial Pathogen Causing American Foulbrood Disease in Honeybees. *Applied and Environmental Microbiology*, *71*(11), 7551–7555. https://doi.org/10.1128/AEM.71.11.7551-7555.2005

Genersch, E., & Otten, C. (2003). The use of repetitive element PCR fingerprinting (rep-PCR) for genetic subtyping of German field isolates of Paenibacillus larvae subsp. larvae. *Apidologie*, *34*(3), 195–206. https://doi.org/10.1051/apido:2003025

Ghorbani-Nezami, S., LeBlanc, L., Yost, D. G., & Amy, P. S. (2015). Phage Therapy is Effective in Protecting Honeybee Larvae from American Foulbrood Disease. *Journal of Insect Science*, *15*(1), 84. https://doi.org/10.1093/jisesa/iev051

Gochnauer, T. (1955). The isolation of a bacteriophage (bacterial virus) from Bacillus larvae. *Bee World*, *36*(September), 101–103. https://doi.org/10.1080/0005772X.1955.11094880

Gurung, M. B., Partap, U., Sharma, H. K., Islam, N and Tamag, N. B. (2012). *Beekeeping Training for Farmers in the Himalayas - Resource Manual for Trainers.* Kathmandu: International Centre for Integrated Mountain Development.

Guttman, B., Raya, R., & Kutter, E. (2005). Basic Phage Biology. In *Bacteriophages: Biology and Applications*.

Hanlon, G. W. (2007). Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *International Journal of Antimicrobial Agents*, *30*(2), 118–128. https://doi.org/10.1016/j.ijantimicag.2007.04.006

Hawkins, C., Harper, D., Burch, D., Änggård, E., & Soothill, J. (2010). Topical treatment of Pseudomonas aeruginosa otitis of dogs with a bacteriophage mixture: A before/after clinical trial. *Veterinary Microbiology*, *146*(3–4), 309–313. https://doi.org/10.1016/j.vetmic.2010.05.014

Hendrix, R. W. (2003). Bacteriophage genomics. *Current Opinion in Microbiology*, *6*(5), 506–511. https://doi.org/10.1016/j.mib.2003.09.004

Hertlein, G., Müller, S., Garcia-Gonzalez, E., Poppinga, L., Süssmuth, R. D., & Genersch, E. (2014). Production of the Catechol Type Siderophore Bacillibactin by the Honey Bee Pathogen Paenibacillus larvae. *PLoS ONE*, *9*(9), e108272. https://doi.org/10.1371/journal.pone.0108272

Heyndrickx, M., Vandemeulebroecke, K., Hoste, B., Janssen, P., Kersters, K., De Vos, P., ... Berkeley, R. C. W. (1996). Reclassification of Paenibacillus (formerly Bacillus) pulvifaciens (Nakamura 1984) Ash et al. 1994, a Later Subjective Synonym of Paenibacillus (formerly Bacillus) larvae (White 1906) Ash et al. 1994, as a Subspecies of P. larvae, with Emended Description. *International Journal of Systematic Bacteriology*, *46*(1), 270–279. https://doi.org/10.1099/00207713-46-1-270

Housby, J. N., & Mann, N. H. (2009). Phage therapy. *Drug Discovery Today*, *14*(11–12), 536–540. https://doi.org/10.1016/j.drudis.2009.03.006

John, W. (2005). Lysogeny, prophage induction, and lysogenic conversion. In *Phages:Their Role in Bacterial Pathogenesis and Biotechnology* (pp. 37–54).

Johnson, B. R. (2010). Division of labor in honeybees: form, function, and proximate mechanisms. *Behavioral Ecology and Sociobiology*, *64*(3), 305–316. https://doi.org/10.1007/s00265-009-0874-7

Johnson, R., & Lynne Corn, M. (2016). Bee health: Background and issues for congress. *Bee Health: Problems for Pollinators and Protection Efforts*, 1–46.

Jończyk-Matysiak, E., Popiela, E., Owczarek, B., Hodyra-Stefaniak, K., Świtała-Jeleń, K., Łodej, N., ... Górski, A. (2020). Phages in Therapy and Prophylaxis of American Foulbrood – Recent Implications From Practical Applications. *Frontiers in Microbiology*, *11*(August), 1–16. https://doi.org/10.3389/fmicb.2020.01913

Krska, D., Ravulapalli, R., Fieldhouse, R. J., Lugo, M. R., & Merrill, A. R. (2015). C3larvin Toxin, an ADP-ribosyltransferase from Paenibacillus larvae. *Journal of Biological Chemistry*, *290*(3), 1639–1653. https://doi.org/10.1074/jbc.M114.589846

Kutateladze, M., & Adamia, R. (2010). Bacteriophages as potential new therapeutics to replace or supplement antibiotics.

Trends in Biotechnology, *28*(12), 591–595. https://doi.org/10.1016/j.tibtech.2010.08.001

Kuzyšinová, K., Mudroňová, D., Toporčák, J., Molnár, L., & Javorský, P. (2016). The use of probiotics, essential oils and fatty acids in the control of American foulbrood and other bee diseases. *Journal of Apicultural Research*, *55*(5), 386–395. https://doi.org/10.1080/00218839.2016.1252067

Kwong, W. K., & Moran, N. A. (2016). Gut microbial communities of social bees. *Nature Reviews. Microbiology*, *14*(6), 374–384. https://doi.org/10.1038/nrmicro.2016.43

Laho, M., Koh, L., Mojžišov, A., Majt, J., & Klaudiny, J. (2018). 10-HDA , A Major Fatty Acid of Royal Jelly , Exhibits pH Dependent Growth-Inhibitory Activity Against Different Strains of Paenibacillus larvae. *Molecules*. https://doi.org/10.3390/molecules23123236

Lamei, S., Stephan, J. G., Nilson, B., Miranda, J. R. De, & Forsgren, E. (2020). Feeding Honeybee Colonies with Honeybee-Specific Lactic Acid Bacteria (Hbs-LAB) Does Not Affect Colony-Level Hbs-LAB Composition or Paenibacillus larvae Spore Levels , Although American Foulbrood Affected Colonies Harbor a More Diverse Hbs-LAB Community. *Microbial Ecology*, 743–755. https://doi.org/10.1007/s00248-019-01434-3

LeBlanc, L., Nezami, S., Yost, D., Tsourkas, P., & Amy, P. S. (2015). Isolation and characterization of a novel phage lysin active against Paenibacillus larvae , a honeybee pathogen. *Bacteriophage*, *5*(4), e1080787. https://doi.org/10.1080/21597081.2015.1080787

Leverentz, B., Conway, W. S., Camp, M. J., Janisiewicz, W. J., Abuladze, T., Yang, M., … Sulakvelidze, A. (2003). Biocontrol of Listeria monocytogenes on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology*, *69*(8), 4519–4526. https://doi.org/10.1128/AEM.69.8.4519-4526.2003

Loc-Carrillo, C., & Abedon, S. T. (2011). Pros and cons of phage therapy. *Bacteriophage*, *1*(2), 111–114. https://doi.org/10.4161/bact.1.2.14590

Lu, T. K., & Collins, J. J. (2007). Dispersing biofilms with engineered enzymatic bacteriophage. *Proceedings of the National Academy of Sciences*, *104*(27), 11197–11202. https://doi.org/10.1073/pnas.0704624104

Meader, E., Mayer, M. J., Steverding, D., Carding, S. R., & Narbad, A. (2013). Evaluation of bacteriophage therapy to control Clostridium difficile and toxin production in an in vitro human colon model system. *Anaerobe*, *22*, 25–30. https://doi.org/10.1016/j.anaerobe.2013.05.001

Merrill, B. ., Fajardo, C. ., Hilton, J. ., Payne, A. ., Ward, A. ., Walker, J. ., … Al., E. (2018). Complete genome sequences of eighteen Paenibacillus larvae phages. *Genome Announc*.

Milbrath, M. (2021). Honey Bee Bacterial Diseases, (Bailey 1974), 277–293. https://doi.org/10.1002/9781119583417.ch22

Miyagi, T., Peng, C. Y. S., Chuang, R. Y., Mussen, E. C., Spivak, M. S., & Doi, R. H. (2000). Verification of oxytetracycline-resistant American foulbrood pathogen Paenibacillus larvae in the United States. *Journal of Invertebrate Pathology*, *75*(1), 95–96. https://doi.org/10.1006/jipa.1999.4888

Monteiro, R., Pires, D. P., Costa, A. R., & Azeredo, J. (2018). Phage Therapy: Going Temperate? *Trends in Microbiology*, *xx*, 1–11. https://doi.org/10.1016/j.tim.2018.10.008

Müller, S., Garcia-Gonzalez, E., Genersch, E., & Süssmuth, R. D. (2015). Involvement of secondary metabolites in the pathogenesis of the American foulbrood of honey bees caused by Paenibacillus larvae. *Natural Product Reports*, *32*(6), 765–778. https://doi.org/10.1039/C4NP00158C

Müller, S., Garcia-Gonzalez, E., Mainz, A., Hertlein, G., Heid, N. C., Mösker, E., … Süssmuth, R. D. (2014). Paenilamicin: Structure and Biosynthesis of a Hybrid Nonribosomal Peptide/Polyketide Antibiotic from the Bee Pathogen Paenibacillus larvae. *Angewandte Chemie International Edition*, *53*(40), 10821–10825. https://doi.org/10.1002/anie.201404572

Murray, E., Draper, L. A., Ross, R. P., & Hill, C. (2021). The Advantages and Challenges of Using Endolysins in a Clinical Setting. *Viruses*, *13*(4), 680. https://doi.org/10.3390/v13040680

Nale, J., Redgwell, T., Millard, A., & Clokie, M. (2018). Efficacy of an Optimised Bacteriophage Cocktail to Clear Clostridium difficile in a Batch Fermentation Model. *Antibiotics*, *7*(1), 13. https://doi.org/10.3390/antibiotics7010013

Nale, J. Y., Spencer, J., Hargreaves, K. R., Buckley, A. M., Trzepin, P., & Douce, G. R. (2016). Bacteriophage Combinations Significantly Reduce Clostridium difficile Growth In Vitro and Proliferation In Vivo. *Antimicrobial Agents and Chemotherapy*, *60*(2), 968–981. https://doi.org/10.1128/AAC.01774-15.Address

Neuendorf, S., Hedtke, K., Tangen, G., & Genersch, E. (2004). Biochemical characterization of different genotypes of Paenibacillus larvae subsp. larvae, a honey bee bacterial pathogen. *Microbiology*, *150*(7), 2381–2390. https://doi.org/10.1099/mic.0.27125-0

Nicolson, S. W. (2009). Water homeostasis in bees, with the emphasis on sociality. *Journal of Experimental Biology*, *212*(3), 429–434. https://doi.org/10.1242/jeb.022343

Nixon, H., & Ribbands, C. (1952). Food transmission within the honeybee community. *Proceedings of the Royal Society of London. Series B - Biological Sciences*, *140*(898), 43–50. https://doi.org/10.1098/rspb.1952.0042

Nobrega, F. L., Costa, A. R., Santos, J. F., Siliakus, M. F., Van Lent, J. W. M., Kengen, S. W. M., … Kluskens, L. D. (2016). Genetically manipulated phages with improved pH resistance for oral administration in veterinary medicine. *Scientific Reports*, *6*(November), 1–12. https://doi.org/10.1038/srep39235

Oliveira, A., Leite, M., Kluskens, L. D., Santos, S. B., Melo, L. D. R., & Azeredo, J. (2015). The First Paenibacillus larvae Bacteriophage Endolysin (PlyPl23) with High Potential to Control American Foulbrood. *PLOS ONE*, *10*(7), e0132095. https://doi.org/10.1371/journal.pone.0132095

Oliveira, A., Melo, L. D. R., Kropinski, A. M., & Azeredo, J. (2013). Complete Genome Sequence of the Broad-Host-Range Paenibacillus larvae Phage phiIBB_Pl23. *Genome Announcements*, *1*(5), e00438-13-e00438-13. https://doi.org/10.1128/genomeA.00438-13

Oliveira, A., Sereno, R., & Azeredo, J. (2010). In vivo efficiency evaluation of a phage cocktail in controlling severe colibacillosis in confined conditions and experimental poultry houses. *Veterinary Microbiology*, *146*(3–4), 303–308. https://doi.org/10.1016/j.vetmic.2010.05.015

Oliver, R. (2021a). Honey Bee Nutrition. In *Honey Bee Medicine for the Veterinary Practitioner* (Vol. 157, pp. 93–123). Wiley. https://doi.org/10.1002/9781119583417.ch8

Oliver, R. (2021b). The Honey Bee Queen. In *Honey Bee Medicine for the Veterinary Practitioner* (pp. 55–71). Wiley. https://doi.org/10.1002/9781119583417.ch5

Pasupuleti, V. R., Sammugam, L., Ramesh, N., & Gan, S. H. (2017). Honey, propolis, and royal jelly: A comprehensive review of their biological actions and health benefits. *Oxidative Medicine and Cellular Longevity*, *2017*, 1–21. https://doi.org/10.1155/2017/1259510

Pellegrini, M. C., Alonso-Salces, R. M., Umpierrez, M. L., Rossini, C., & Fuselli, S. R. (2017). Chemical Composition, Antimicrobial Activity, and Mode of Action of Essential Oils against Paenibacillus larvae, Etiological Agent of American Foulbrood on Apis mellifera. *Chemistry and Biodiversity*, *14*(4). https://doi.org/10.1002/cbdv.201600382

Pellegrini, M. C., Zalazar, L., Fuselli, S. R., & Ponce, A. G. (2017). Inhibitory action of essential oils against proteases activity of Paenibacillus larvae , the etiological agent of American Foulbrood disease. *Spanish Journal of Agricultural Research*, *15*(4). https://doi.org/10.5424/sjar/2017154-10785

Pires, D. P., Cleto, S., Sillankorva, S., Azeredo, J., & Lu, T. K. (2016). Genetically Engineered Phages: a Review of Advances over the Last. *Microbiology and Moleculat Biology Reviews*, *80*(3), 523–543. https://doi.org/10.1128/MMBR.00069-15.Address

Pires, D. P., Costa, A. R., Pinto, G., Meneses, L., & Azeredo, J. (2020). Current challenges and future opportunities of phage therapy. *FEMS Microbiology Reviews*, *44*(6), 684–700. https://doi.org/10.1093/femsre/fuaa017

Pires, D. P., Monteiro, R., Mil-Homens, D., Fialho, A., Lu, T. K., & Azeredo, J. (2021). Designing P. aeruginosa synthetic phages with reduced genomes. *Scientific Reports*, *11*(1), 1–10. https://doi.org/10.1038/s41598-021-81580-2

Popova, A., Valerianov, T., & Toschkoff, A. . (1976). Bacteriophage of Bacillus larvae. *Comptes Rendus de l' Academie Bulgare Des Sciences*, *28*(2), 249–252.

Poppinga, L., & Genersch, E. (2015). Molecular pathogenesis of American Foulbrood: how Paenibacillus larvae kills honey bee larvae. *Current Opinion in Insect Science*, *10*, 29–36. https://doi.org/10.1016/j.cois.2015.04.013

Poppinga, L., Janesch, B., Fünfhaus, A., Sekot, G., Garcia-Gonzalez, E., Hertlein, G., … Genersch, E. (2012). Identification and Functional Analysis of the S-Layer Protein SplA of Paenibacillus larvae, the Causative Agent of American Foulbrood of Honey Bees. *PLoS Pathogens*, *8*(5), e1002716. https://doi.org/10.1371/journal.ppat.1002716

Potera, C. (2013). Phage renaissance: new hope against antibiotic resistance. *Environmental Health Perspectives*, *121*.

Powell, J. E., Martinson, V. G., Urban-Mead, K., & Moran, N. A. (2014). Routes of Acquisition of the Gut Microbiota of the Honey Bee Apis mellifera. *Applied and Environmental Microbiology*, *80*(23), 7378–7387. https://doi.org/10.1128/AEM.01861-14

Radcliffe, R. W. (2021). The Superorganism and Herd Health for the Honey Bee. *Honey Bee Medicine for the Veterinary Practitioner*, 21–31. https://doi.org/10.1002/9781119583417.ch2

Radcliffe, R. W., & Seeley, T. D. (2021). Looking to Nature to Solve the Health Crisis of Honey Bees. In *Honey Bee Medicine for the Veterinary Practitioner* (pp. 1–20). Wiley. https://doi.org/10.1002/9781119583417.ch1

Rauch, S., Ashiralieva, A., Hedtke, K., & Genersch, E. (2009). Negative Correlation between Individual-Insect-Level Virulence and Colony-Level Virulence of Paenibacillus larvae, the Etiological Agent of American Foulbrood of Honeybees. *Applied and Environmental Microbiology*, *75*(10), 3344–3347. https://doi.org/10.1128/AEM.02839-08

Raymann, K. (2021). Honey Bee Microbiota and the Physiology of Antimicrobial Resistance. In *Honey Bee Medicine for the Veterinary Practitioner* (pp. 125–134). Wiley. https://doi.org/10.1002/9781119583417.ch9

Raymann, K., & Moran, N. A. (2018). The role of the gut microbiome in health and disease of adult honey bee workers. *Current Opinion in Insect Science*, *26*, 97–104. https://doi.org/10.1016/j.cois.2018.02.012

Ribeiro, H. G., Correia, R., Moreira, T., Vilas Boas, D., Azeredo, J., & Oliveira, A. (2019). Bacteriophage biodistribution and infectivity from honeybee to bee larvae using a T7 phage model. *Scientific Reports*, *9*(1), 620. https://doi.org/10.1038/s41598-018-36432-x

Ribeiro, H. G., Melo, L. D. R., Oliveira, H., Boon, M., Lavigne, R., Noben, J.-P., … Oliveira, A. (2019). Characterization of a new podovirus infecting Paenibacillus larvae. *Scientific Reports*, *9*(1), 20355. https://doi.org/10.1038/s41598-019-56699-y

Rohwer, F., & Edwards, R. (2002). The phage proteomic tree: a genome-based taxonomy for phage. *Journal of Bacteriology*,

184(16), 4529–4535. https://doi.org/10.1128/JB.184.16.4529-4535.2002

Santos, S. B., Oliveira, A., Melo, L. D. R., & Azeredo, J. (2019). Identification of the first endolysin Cell Binding Domain (CBD) targeting Paenibacillus larvae. *Scientific Reports*, *9*(1), 2568. https://doi.org/10.1038/s41598-019-39097-2

Schild, H. A., Fuchs, S. W., Bode, H. B., & Grünewald, B. (2014). Low-molecular-weight metabolites secreted by Paenibacillus larvae as potential virulence factors of American foulbrood. *Applied and Environmental Microbiology*, *80*(8), 2484–2492. https://doi.org/10.1128/AEM.04049-13

Simone-Finstrom, M. (2017). Social Immunity and the Superorganism: Behavioral Defenses Protecting Honey Bee Colonies from Pathogens and Parasites. *Bee World*, *94*(1), 21–29. https://doi.org/10.1080/0005772X.2017.1307800

Smirnova, N. I. (1953). Isolation and the use of a bacteriophage of Bacillus larvae in the diagnosis of American foulbrood. *Sbornik Nauchnykh Trudov Leningradskogo Instituta Usovershenstvovaniya Veterinarykh Vrachel (in Russiun)*, *9*, 85–88.

Sood, S., Steinmetz, H., Beims, H., Mohr, K. I., Stadler, M., Djukic, M., … Müller, R. (2014). Paenilarvins: Iturin family lipopeptides from the honey bee pathogen Paenibacillus larvae. *Chembiochem : A European Journal of Chemical Biology*, *15*(13), 1947–1955. https://doi.org/10.1002/cbic.201402139

Spricigo, D. A., Bardina, C., Cortés, P., & Llagostera, M. (2013). Use of a bacteriophage cocktail to control Salmonella in food and the food industry. *International Journal of Food Microbiology*, *165*(2), 169–174. https://doi.org/10.1016/j.ijfoodmicro.2013.05.009

Stahly, D. P., Alippi, A. M., Bakhiet, N., Campana, C. F., Novak, C. C., & Cox, R. (1999). PPL1c, a Virulent Mutant Bacteriophage Useful for Identification of Paenibacillus larvae subspecies larvae. *Journal of Invertebrate Pathology*, *74*(3), 295–296. https://doi.org/10.1006/jipa.1999.4893

Stamereilers, C., Fajardo, C., Walker, J., Mendez, K., Castro-Nallar, E., Grose, J., … Tsourkas, P. (2018). Genomic Analysis of 48 Paenibacillus larvae Bacteriophages. *Viruses*, *10*(7), 377. https://doi.org/10.3390/v10070377

Stephan, J. G., Riesbeck, K., & Miranda, J. R. De. (2019). Honeybee-Specific Lactic Acid Bacterium Supplements Have No Effect on American Foulbrood-Infected Honeybee Colonies. *Applied and Environmental Microbiology*.

Sulakvelidze, A., Alavidze, Z., & Morris, J. G. (2001). Bacteriophage Therapy. *Antimicrobial Agents and Chemotherapy*, *45*(31), 649–659. https://doi.org/10.1128/AAC.45.3.649

Summers, W. C., Stent, G., & Twort, F. (2012). The strange history of phage therapy. *Landes Bioscience*, *2*(2), 130–133. https://doi.org/10.4161/bact.20757

Tan, S. Y., & Tatsumura, Y. (2015). Alexander Fleming (1881–1955): Discoverer of penicillin, *56*(7), 366–367. https://doi.org/10.11622/smedj.2015105

Tsourkas, P. K. (2020). Paenibacillus larvae bacteriophages: obscure past, promising future. *Microbial Genomics*, *6*(2). https://doi.org/10.1099/mgen.0.000329

Tsourkas, P. K., Yost, D. G., Krohn, A., LeBlanc, L., Zhang, A., Stamereilers, C., & Amy, P. S. (2015). Complete Genome Sequences of Nine Phages Capable of Infecting Paenibacillus larvae , the Causative Agent of American Foulbrood Disease in Honeybees. *Genome Announcements*, *3*(5), e01120-15. https://doi.org/10.1128/genomeA.01120-15

Turner, D., Kropinski, A. M., & Adriaenssens, E. M. (2021). A roadmap for genome-based phage taxonomy. *Viruses*, *13*(3), 1–10. https://doi.org/10.3390/v13030506

Turner, M., Tremblay, O., Heney, K. A., Lugo, M. R., Ebeling, J., Genersch, E., & Merrill, A. R. (2020). Characterization of C3larvinA, a novel RhoA-targeting ADP-ribosyltransferase toxin produced by the honey bee pathogen, Paenibacillus larvae. *Bioscience Reports*, *40*(1), 5100–5116. https://doi.org/10.1042/BSR20193405

Twort, F. W. (1915). An investigation on the nature of ultra-microscopic viruses. *The Lancet*, *186*(4814), 1241–1243. https://doi.org/10.1016/S0140-6736(01)20383-3

Ueno, Y., Yoshida, E., Misumi, W., Watando, E., Suzuki, K., Hirai, Y., … Takamatsu, D. (2018). Population structure and antimicrobial susceptibility of Paenibacillus larvae isolates from American foulbrood cases in Apis mellifera in Japan. *Environmental Microbiology Reports*, *10*(2), 210–216. https://doi.org/10.1111/1758-2229.12623

Vojvodic, S., Rehan, S. M., & Anderson, K. E. (2013). Microbial Gut Diversity of Africanized and European Honey Bee Larval Instars. *PLoS ONE*, *8*(8), 1–11. https://doi.org/10.1371/journal.pone.0072106

Walker, J. K., Merrill, B. D., Berg, J. A., Dhalai, A., Dingman, D. W., Fajardo, C. P., … Tsourkas, P. K. (2018). Complete genome sequences of Paenibacillus larvae phages BN12, Dragolir, Kiel007, Leyra, Likha, Pagassa, PBL1c, and Tadhana. *Genome Announcements*, *6*(24), 12–13. https://doi.org/10.1128/genomeA.01602-17

Wilson, W. T. (1971). Resistance to American foulbrood in honey bees. XI. Fate of Bacillus larvae spores ingested by adults. *Journal of Invertebrate Pathology*, *17*(2), 247–255. https://doi.org/10.1016/0022-2011(71)90099-1

Woodrow, A. W. (1942). Susceptibility of honeybee larvae to individual inoculations with spores of *Bacillus* larvae. *Journal of Economic Entomology*, *35*(6), 892–895. https://doi.org/10.1093/jee/35.6.892

Woodrow, A. W., & Holst, E. C. (1942). The mechanism of colony resistance to American foulbrood. *Journal of Economic Entomology*, *35*(3), 327–330. https://doi.org/10.1093/jee/35.3.327

Wu, X. Y., Chin, J., Ghalayini, A., & Hornitzky, M. (2005). Pulsed-field gel electrophoresis typing and oxytetracycline sensitivity of Paenibacillus larvae subsp. larvae isolates of Australian origin and those recovered from honey imported from

Argentina. *Journal of Apicultural Research*, *44*(2), 87–92. https://doi.org/10.1080/00218839.2005.11101155

Yost, D. G., Chang, C., LeBlanc, L., Cassin, E., Peterman, C., Rai, P., ... Tsourkas, P. K. (2018). Complete Genome Sequences of Paenibacillus larvae Phages Halcyone, Heath, Scottie, and Unity from Las Vegas, Nevada. *Microbiology Resource Announcements*, *7*(12), 1–2. https://doi.org/10.1128/MRA.00977-18

Yost, D. G., Tsourkas, P., & Amy, P. S. (2016). Experimental bacteriophage treatment of honeybees (Apis mellifera) infected with Paenibacillus larvae, the causative agent of American foulbrood disease. *Bacteriophage*, *6*(1), e1122698. https://doi.org/10.1080/21597081.2015.1122698

Yue, D., Nordhoff, M., Wieler, L. H., & Genersch, E. (2008). Fluorescence in situ hybridization (FISH) analysis of the interactions between honeybee larvae and Paenibacillus larvae, the causative agent of American foulbrood of honeybees (Apis mellifera). *Environmental Microbiology*, *10*(6), 1612–1620. https://doi.org/10.1111/j.1462-2920.2008.01579.x

Zheng, H., Powell, J. E., Steele, M. I., Dietrich, C., & Moran, N. A. (2017). Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(18), 4775–4780. https://doi.org/10.1073/pnas.1701819114

Žugelj, A., Papić, B., Zdovc, I., Zajc, U., Golob, M., Avberšek, J., & Kušar, D. (2021). ERIC and WGS Typing of Paenibacillus larvae in Slovenia: Investigation of ERIC I Outbreaks. *Insects*, *12*(4), 362. https://doi.org/10.3390/insects12040362

CHAPTER 2: BACTERIOPHAGE BIODISTRIBUTION AND INFECTIVITY FROM HONEYBEE TO BEE LARVAE USING A T7 PHAGE MODEL

This chapter was based on the following paper:

Ribeiro HG*, Correia R, Moreira T, Vilas Boas D, Azeredo J, Oliveira A. Bacteriophage biodistribution and infectivity from honeybee to bee larvae using a T7 phage model. Sci Rep. 2019;9(1):620. https://doi.org/10.1038/s41598-018-36432-x

Abstract

Bacteriophages (phages) or viruses that specifically infect bacteria have widely been studied as biocontrol agents against animal and plant bacterial diseases. They offer many advantages compared to antibiotics. The American Foulbrood disease (AFB) is a bacterial disease affecting honeybee larvae caused by *Paenibacillus larvae.* Phages can be very significant in fighting it mostly due to European restrictions to the use of antibiotics in beekeeping. New phages able to control *P. larvae* in hives have already been reported with satisfactory results. However, the efficacy and feasibility of administering phages indirectly to larvae through their adult workers only by providing phages in bees' feeders has never been evaluated. This strategy is considered herein the most feasible as far as hive management is concerned. This *in vivo* study investigated the ability of a phage to reach larvae in an infective state after oral administration to honeybees. The screening (by direct plaque forming units (PFU) count) and quantification (by quantitative PCR) of the phage in bee organs and in larvae after ingestion allowed us to conclude that despite 10^4 phages reaching larvae only an average of 32 were available to control the spread of the disease. The fast inactivation of many phages in royal jelly could compromise this therapeutic approach. The protection of phages from hive-derived conditions should be thus considered in further developments for AFB treatment.

2.1 Introduction

Bacteriophages (phages) are viruses that exclusively infect bacteria and are highly specific for their hosts. They take advantage of bacteria biosynthetic machinery by directing it toward the synthesis of more phages able to induce the bacterial lysis and phage release to the environment. New infection cycles will be triggered as soon as phages successfully reach available hosts nearby. The binding of phage virions to specific bacterial receptors with subsequent bacterial death makes them highly specific antibacterial agents (Abedon et al., 2011). Their self-replicating properties enable that efficient concentrations of phage particles are achieved at the site of infection at curative doses (Drulis-Kawa, Majkowska-Skrobek, & Maciejewska, 2015). Phages are also recognised by the inability to infect eukaryotic cells showing no toxicity to plants, animals or humans (Atterbury, 2009). It is estimated that 10^{31} phages occur in the biosphere (Rohwer & Edwards, 2002).

All these described features make phages a promising strategy to control bacterial infections, including American Foulbrood disease (AFB). AFB is one of the most devastating bacterial diseases affecting honeybee larvae worldwide (Hansen & Brødsgaard, 1999). This contagious disease is caused by the vegetative form of the Gram-positive bacterium *Paenibacillus larvae* and is easily spread through their highly resilient spores. The sporulated form of the bacteria withstand extreme temperatures and exposure to disinfectants, tolerate antibiotics and is able to remain dormant for years (Lindström, Korpela, & Fries, 2008).

The treatment of AFB represents an important challenge to the pharmaceutical industry as the hive-derived products (honey, propolis, royal jelly, bee venom and bee pollen) cannot be commercialised if contaminated with antibiotics (Pasupuleti et al., 2017) (EC regulation 2377/90 and further amendments (European Parliament and the Council of the European Union, 2010)). Furthermore, bacterial resistance to the commonly prescribed antibiotics (Alippi, López, Reynaldi, Grasso, & Aguilar, 2007; Tian, Fadhil, Powell, Kwong, & Moran, 2012) contributes to reduce the available alternatives to the conventional burning of hives for *P. larvae* control.

AFB infection process begins when adult bees provide spore-contaminated food to their larvae that become infected during the first instars (around 36 hours after the egg hatching) (Karl Crailsheim & Riessberger-Gallé, 2001; Genersch, 2010a; Genersch et al., 2005; Woodrow, 1942). As soon as spores arrive to larvae midgut they germinate into vegetative cells and massively proliferate (Djukic et al., 2014; Genersch et al., 2005). In the last stage of the infection *P. larvae* breach the midgut epithelium and invade the larval haemocoel resulting in larval death (Yue et al., 2008). After depletion of the nutrients *P.*

larvae starts sporulation and billions of spores are released inside the hives that easily spread across apiaries (Alvarado et al., 2013).

Efforts have been made to explore the potential of phage therapy for treating AFB by evaluating phage action against *P. larvae* (Beims et al., 2015; Brady et al., 2017; Ghorbani-Nezami et al., 2015; A. Oliveira et al., 2013; Yost et al., 2016). Recently published studies report the use of spore-infected laboratory-raised larvae to evaluate phage effectiveness in decreasing larvae mortality. In those studies larvae were fed directly with phages eventually simulating the spray administration to larvae combs. The success of a prophylactic treatment of larvae with phages before spore infection was assessed (Ghorbani-Nezami et al., 2015; Yost et al., 2016), but no consensual results were obtained concerning the efficacy of treating larvae after infection (Beims et al., 2015; Ghorbani-Nezami et al., 2015; Yost et al., 2016). More recently, in tests performed in experimental hives Brady et al. (2017) reported that larvae sprayed with phages were effectively protected and rescued from *P. larvae* infections (Brady et al., 2017). The phage delivery in the adult bee food might though be a more feasible strategy toward hive management causing lower phage waste. Hence, a thorough assessment of phage biodistribution and bioavailability from adult bee to young larvae was performed in this work relying on the bee social organization for brood rearing.

2.2 Material and methods

2.2.1 Phage production

E. coli BL21 (Stratagene) was the strain used as T7, T1 and T4 phage propagation strain. T7 phage was gently provided by J. Molineux (University of Texas), and T1 and T4 phages by Stan Brouns (Delft University of Technology). Bacteria were cultured at 37 °C overnight (O/N) in Tryptic Soy Broth (TSB, VWR) or Tryptic Soy Agar medium (TSA; TSB containing 1.5% (w/v) agar, NZYTech). For phage propagation, 5 μL of phage suspension were spread evenly on host bacterial lawns using a paper strip and incubated O/N at 37 °C. Then, 3 mL of SM Buffer (5.8 $g.L^{-1}$ NaCl, 2 $g.L^{-1}$ $MgSO_4.7H_2O$, 50 $mL.L^{-1}$ 1 M Tris-HCl pH 7.5, VWR) were added to each plate and re-incubated O/N at 4 °C with gentle stirring (50 rpm on a PSU-10i Orbital Shaker (Biosan)). The floating liquid was collected, centrifuged (10 min, 9000 ×g, 4 °C), and purified with 1:4 (v/v) chloroform followed by filter sterilization (PES, GE Healthcare, 0.22 μm). Phage suspensions were stored at 4 °C until use.

2.2.2 Phage viability in sucrose and royal jelly (RJ)

Before *in vivo* experiments, envisaging honeybees feeding, the effect of 50% (w/v) sucrose solution in T7 phage viability was evaluated. After field trials, in order to support *in vivo* results, the influence of a commercially available RJ (pH 4.0) (Apiguarda, Portugal) in T7 phage infectivity was monitored with time. This was done also for T1 and T4 phages to support data obtained for T7 phage. Universal Buffer solutions (150 mM KCl, 10 mM KH_2PO_4, 10 mM Na-Citrate and 10 mM H_3BO_3) adjusted with HCl to have pH of 3.5, 4.0 and 4.5 were prepared in order to assess phage behaviour in the pH ranges reported for RJ, including the used herein. The final phage concentration was 10^9 $PFU.mL^{-1}$. The incubation in sucrose was performed at room temperature (RT) and the incubation in RJ and buffered solutions at 37 °C with 5% CO_2, to mimic hive conditions. Samples were collected at 0, 6, 9, 12 and 24 hours in the first case and at 0, 1, 3 and 6 hours in the others. In each time point, three independent samples were taken and the titration was performed as described above.

2.2.3 Screening for T7 phage and *E. coli* hosts strains in experimental hives

In order to assure that the phages present in hives were only provided by artificial feeding the screening for other phages able to infect the *E. coli* strain used in this study was performed. The presence of other bacterial strains that could be sensitive to T7 phage was also examined in the colonies. These procedures intended to avoid phage overestimation. For that, samples were collected from empty and brood combs of each colony, using swabs soaked in 0.9% (w/v) NaCl. For phage detection, swabs were

immersed in an *E. coli* Bl21 early-grown suspension (about 6 hours), incubated O/N at 37 °C (120 rpm) and after that, the suspension was filter-sterilised (0.22 µm, PES). A drop of 10 µL of the filtrate was placed onto 0.6% TSA agar previously inoculated with 100 µL of an *E. coli* BL21 suspension, and re-incubated at 37 °C, O/N. For *E. coli* detection, swabs were streaked in TSA and MacConkey agar (Merck-chemicals), followed by an O/N incubation at 37 °C. Plates were searched for colonies and in the case of positive results, colonies were suspended in TSB and tested for phage sensitivity as described.

2.2.4 Biodistribution assay

2.2.4.1 Experimental model

The experimental apiary used for this *in vivo* experiment was located in the north of Portugal (Vila Nova de Famalicão) where six different colonies of *Apis mellifera* with the same dimension and under the same development state were housed.

For phage administration, 1×10^9 PFU.mL^{-1} suspended in a 50% (w/v) sucrose solution were provided to bees' feeders. After 24 hours, 30 adult bees and 15 larvae from 1st- 3rd instar were collected from each colony (larvae were carefully grafted into a microtube together with the surrounding RJ with the aid of a larvae-picking tool).

Both adult bees and larvae were carefully washed 3× with saline solution (0.9% (w/v) NaCl) before processing. Bees were treated as follows: after removing their wings and legs they were divided in three parts: head, thorax and abdomen. The content of each part was carefully removed with the aid of two sterile forceps: heads and thoraxes' content (H&T) were mixed together; guts were removed from the abdomens (Abd) and treated separately. Larvae embedded in RJ were weighted (L_RJ). After the first washing the decanted supernatant (which volume was recorded as first wash) was recovered for phage titration and larvae weight (L) was recorded. The dilution rate (w/v) of RJ (obtained from L_RJ (g) – L (g)) in first wash was assessed for further calculations.

All the samples, except RJ, were well homogenised in 0.9% (w/v) NaCl (the volume used was recorded for further calculations) with glass beads, by vortexing. Samples were stored at 4 °C for no more than 30 minutes for further analysis. In a previous assay, T7 phage suspended in SM buffer was similarly homogenised to exclude the possibility of phage viability loss due to the sample processing.

2.2.4.2 Determination of viable T7 phage in bees and larvae

Phage counts were performed in 50 µL of bee (H&T and Abd) and larvae homogenates. Each sample was mixed with chloroform (5:1 (v/v)) homogenised by vortexing and centrifuged at 8000 ×*g*, 4 °C, for 3 minutes. The upper phase was collected carefully to a new tube and phage titration was done based on the double agar overlay technique (Adams, 1959), as previously described. This analysis was also performed in the first larvae washing (diluted RJ).

2.2.4.3 DNA extraction and purification from biological samples

T7 phage DNA present in the above treated samples was assessed by quantitative PCR (qPCR) in a CFX96 thermal cycler (Bio-Rad) and for that, two different genes with published sequences were targeted in separate reactions: the T7 major capsid protein (MCP) gene for phage detection and the *E. coli LacZ* gene for the internal amplification control (IAC), used to avoid false negative results and to assess phage DNA purification efficiency. Primers (**Table 3**) were designed using SnapGene™ software (version 1.1.3) (www.snapgene.com).

The DNA was purified from the previously homogenised samples of H&T, Abd, larvae and from diluted RJ. Before DNA extraction, samples were supplemented with 25 ng.µL^{-1} of the IAC template (a 3075 bp purified amplicon originary from *E. coli LacZ* gene). Zymo Quick-DNA™ Viral Kit was used for DNA purification, with some modifications relatively to the manufacturer instructions: a 3 hours period of incubation with the supplied Lysis Buffer and the elution in 15 µL of the supplied Storage Buffer.

2.2.4.4 T7 phage quantification in bees and larva

In order to quantify the IAC concentration present in the purified DNA, a standard curve was obtained (Cq_{IAC} = -3.781 × LOG [IAC] (ng.µL^{-1}) + 8.369 (Cq = quantification cycle)) using several concentrations of the *LacZ* amplicon (5.5, 0.55, 0.11, 0.055 ng.µL^{-1}). For that 5 µL of SsoFast™ EvaGreen (BioRad), 2 µL of DNA template, 1 µL of a 5 µM LacZ (IAC) primer mix and HyPure™ Molecular Biology Grade water (GE, Healthcare) up to 10 µL were mixed and run for 3 minutes at 95 °C followed by 40 cycles of 10 seconds at 95 °C, 10 seconds at 58 °C, and 10 seconds at 65 °C (the melt curve was generated by heating from 65 to 95 °C with increments of 1 °C, 5 seconds dwell time).

The assessment of the DNA extraction efficacy was obtained through **Equation 1** that was used to normalise T7 phage concentration obtained from biological samples.

Equation 1: 100 – ((25 ng.µL^{-1} – $[IAC]_{sample}$ ng.µL^{-1}) / 25 ng.µL^{-1})*100

To assess the amplification efficiency of the primer pair T7 MCP another standard curve was defined: Cq_{MCP} = -3.486 × LOG [T7 MCP] (ng.µL^{-1}) + 11.467). T7 phage commercial DNA (BIORON) serial dilutions (0.3; 0.1; 0.03; 0.01 ng.µL^{-1}) were used as template for the qPCR reaction mixture. The annealing temperature, in this case was 54 °C.

In order to estimate T7 phage concentration (PFU.mL^{-1}) in the treated samples, the DNA of five serial dilutions of a 10^8 PFU.mL^{-1} T7-phage suspension was purified (Zymo Quick-DNA™ Viral Kit), and used as template for qPCR analysis (T7 MCP primers). The standard curve equation was obtained (**Equation 2**). A negative control using HyPure™ water was included in each reaction and three replicates of each condition were analysed in all the assays.

Equation 2: Cq_{MCP} = -3.3686 × LOG [T7 phage] (PFU.mL^{-1}) + 43.06

Table 3: Primer sequences used in qPCR, amplicons' size of the PCR products and amplification efficiency of qPCR reactions.

Target gene	Sequence (5'–3')	Amplicon size (bp)	Amplification Efficiency (%)
T7 MCP	F: CCGCAACGTTATGGGCTTTG R: CTCACCTTTATTGGCAGGGAAG	119	93.6
LacZ (IAC)	F: AGCGAAACCGCCAAGACTGTTA R: GTGGATGAAGACCAGCCCTTCC	135	83.6

2.2.4.5 Immunohistochemical targeting of T7 phage in larvae bees

For preparing samples for sectioning 10 larvae were collected from the experimental hive before the beginning of the *in vivo* experiment and used as negative controls. The treated group was composed on 30 larvae picked 24 hours after phage administration. For tissue fixation, all the larvae were previously perfused: 10% (v/v) buffered formalin was introduced with the aid of a perfusion needle. After 24 hours, samples were routinely processed in an automated system and embedded in paraffin. Then, sequential sections of 4 µm were made in a paraffin microtome (Microm HM335E) and placed in adhesive microscope slides (Superfrost®, Sigma).

Before immunohistochemical procedures, sections were dewaxed through immersion in xylene (Fisher Chemical, Loughborough, UK) and sequentially re-hydrated in 100%, 80%, and 50% (v/v) ethanol (Panreac). After rinsed with distilled water slides were allowed to air dry.

Prior to incubation with specific antibodies samples were permeabilised with 0.1% Triton™ X-100 (Sigma) in Phosphate Buffered Saline 1× (PBS, Sigma) for about 10 minutes. Tissue samples were blocked with 5% (w/v) bovine serum albumin (BSA, Sigma) in Tris Buffered Saline with 0.1% (v/v) Tween

20 1× (TBST) and incubated at RT for 90 minutes. The samples were washed 3× with TSBT 1× for 5 minutes.

The incubation with the primary antibody, T7 tag Polyclonal antibody (Invitrogen) (1:1000 in 1% BSA) was added to slides and incubated at 4 °C O/N, in a dark and humid environment. After rinsed 3× with TBST 1×, the Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody conjugated with Alexa Fluor® 488 (Invitrogen) (1:40 in 0.2% BSA) was added and it was allowed to incubate in the dark for 90 minutes at RT.

The samples were rinsed with TBST 1× and mounted with one drop of Vectashield® mounting media (Vector Laboratories). The tissue slides were covered with coverslips and observed by fluorescence microscopy, using a BX51 microscope (Olympus Portugal SA, Porto, Portugal) coupled with a DP71 digital camera and two sets of filters: FITC – 470-490/520 and TRITC – 530-550/590 (Olympus). All images were acquired using the Olympus cellSens software. Phages were examined in 100-fold enlarged images and identified in larvae in 10-fold enlarged reconstructed images.

Control samples were included. For the positive control, a drop of T7 phage suspension (1×10^8 $PFU.mL^{-1}$) was placed on a microscope slide and incubated at 60 °C for 15 minutes. After that, the dried phage drop was incubated with 4% (v/v) paraformaldehyde (Thermo Fisher Scientific) for 10 minutes at RT, followed by 50% (v/v) ethanol for 10 minutes and allowed to air dry. It was then subjected to the incubation process with primary and secondary antibodies as described above.

2.2.4.6 Statistical analysis

The statistical analysis of the results was performed using GraphPad Prism 6. Mean and standard deviations (SD) were determined for the independent experiments and the results were presented as mean ± SD. Results were compared using Two-way ANOVA, with Turkey's multiple comparison statistical test. All tests were performed with a confidence level of 95%. Differences were considered statistically different if $p \leq 0.05$ (95% confidence interval).

2.3 Results

2.3.4 Preliminary *in vitro* analysis

Before undertaking the *in vivo* assays the T7 phage stability in a 50% (w/v) sucrose solution (routinely used for feeding bees) was assessed *in vitro* for 24 hours. The results revealed that, at least in this time period, the phage viability was not affected (**Figure 7**). In order to prevent phage overestimation in further analysis, the screening for phages infecting *E. coli* BL21 (the bacterial T7 host that if present is able to amplify the phage) and for other T7 phage host strains was undertaken in samples obtained from experimental colonies. Both types of analysis revealed the absence of other hosts or phages in the hive.

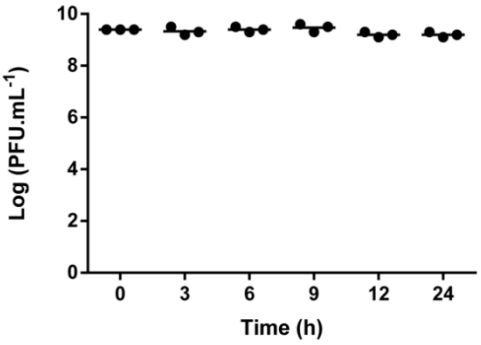

Figure 7: Effect of 50% (w/v) sucrose on T7 phage concentration (PFU.mL^{-1}). Data shows each of the three independent assays (dark circle) and the average (line). LOD (Limit of Detection) = 2 Log.

2.3.5 Biodistribution assay

In vivo assays were conducted in 6 different bee colonies. Adult bees were fed with a T7 phage suspension prepared in 50% (w/v) sucrose. After 24 hours, larvae were grafted – together with the surrounding RJ that was removed from larvae by washing – and stored. Adult bees were also collected, separated into abdomens (Abd), heads (H) and thoraxes (T) and dissected. Heads and thoraxes (H&T) were then mixed as they hold together the part before the foregut (crop). Before that, in order to target only phages present inside their organisms, both adult bees and larvae were washed with saline solution (0.9% (w/v) NaCl). No phage viability loss related with the sample homogenisation with glass beads was observed.

The biodistribution of T7 in adult bees and in bee larvae was assessed by enumeration of the plaque forming units (PFU) in *E. coli* BL21 lawns, which indicates viable phages present inside the organism ("viable phage") and by qPCR, which gives a quantitative estimation of the total amount of phages ("total phage") (**Figure 8**). Results obtained by PFU count revealed that the average amount of viable phages detected per Abd of adult bee (2.8×10^5 PFU/bee) was significantly higher ($p<0.05$) than

per H&T (175 PFU/bee). The average quantity of phages per larvae was 32 PFU/larvae, lower than the observed in H&T, but this difference was not statistically significant (p>0.05).

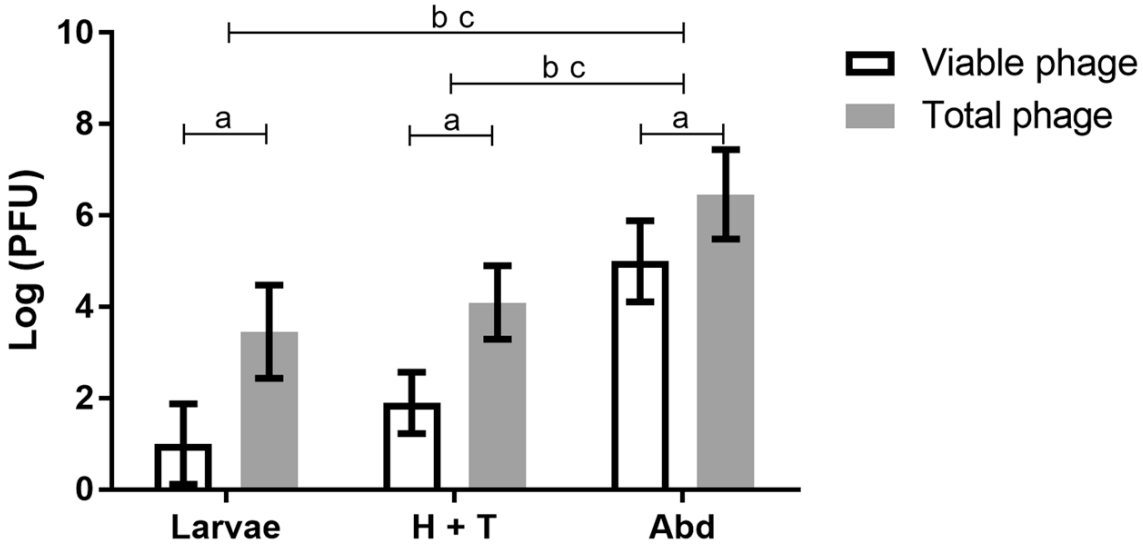

Figure 8: T7 phage (PFU) per section of adult bee (H&T and Abd) and per bee larva, 24 hours after providing phage (10^9 PFU.mL^{-1} in 50% (w/v) sucrose) in bee's feeders. Each column represents the mean of six independent *in vivo* experiments (n=6) and error bars indicate the standard deviation (SD). Statistical significance (p<0.05) is indicated above the columns by "a", "b" or "c": a - differences between viable and total phage; b - differences considering viable phage. c - differences considering total phage.

The amount of phages per bee section or per larva detected by qPCR (calculated by **Equation 2** of section 2.2.4.4) was higher than by direct PFU enumeration (p<0.05) (**Figure 8**). Both methods revealed the same average trend between samples: the amount of phages recorded in the Abd (1.2×10^7 phage/bee) was higher than in the H&T (4.2×10^4 phage/bee) and higher than in the larvae (1.4×10^4 phage/larvae). The two latter groups revealed again no meaningful differences between them (p>0.05).

In each qPCR reaction an IAC was used to avoid false negative results and to assess phage DNA purification efficiency. It should be mentioned that the rate of IAC DNA recovery after sample treatment was lower in H&T and Abd. Therefore records for total phage recovery might be less accurate in adult bee than in larvae.

When surrounding larvae RJ was analysed no viable phages were present. Nevertheless, in average, 8.3×10^6 phage.mL^{-1} were detected by qPCR. The effect of RJ in phage viability was assessed through the incubation of phage in a commercial RJ. Phage particles did not persist infective for more than 3 hours (**Figure 9**). The assumption that this inactivation could probably be due to the low pH of RJ led us to the monitoring of the phage concentration using an Universal buffer solution at pH 4.0 (the same pH of RJ). T7 phage showed a higher tolerance to the buffered solution comparatively to RJ dropping only about 2 Log PFU.mL^{-1} in 6 hours. The same *in vitro* assay was performed for two other well-described

phages from different taxonomic genus T1 and T4, in order to support the interpretation of T7 phage data (**Figure 9**). Both phages revealed to be more stable in RJ than T7 phage, however, the latter preserved its viability for longer comparatively to T1 phage in the buffered solution at pH 4.0. After 6 hours in RJ, T1 and T4 phage decreased about 5 and 2 Log PFU.mL^{-1} respectively ($p<0.05$). Besides pH 4.0, the stability of these phages under pH 3.5 and 4.5 (also reported for RJ) (Barnutiu et al., 2011) was assessed. Similar results were obtained: T4 was the most stable phage followed by T7 and then by T1 phage (**Figure 10**).

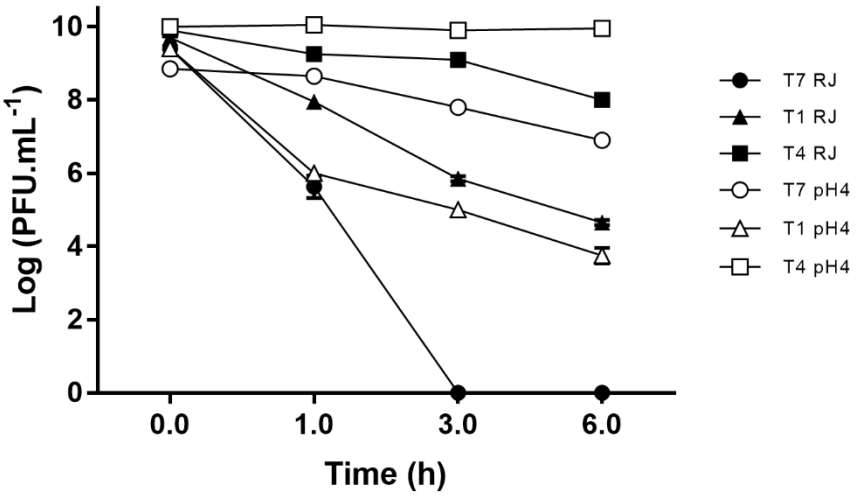

Figure 9: Effect of commercial Royal Jelly (pH 4.0) on the viability of T7 (dark circle), T1 (dark triangle) and T4 (dark square) phages (PFU.mL^{-1}). The control in Universal buffer at pH 4.0 is also plotted for each phage (correspondent white symbols). Limit of Detection = 2 Log; statistical significance, $p<0.05$.

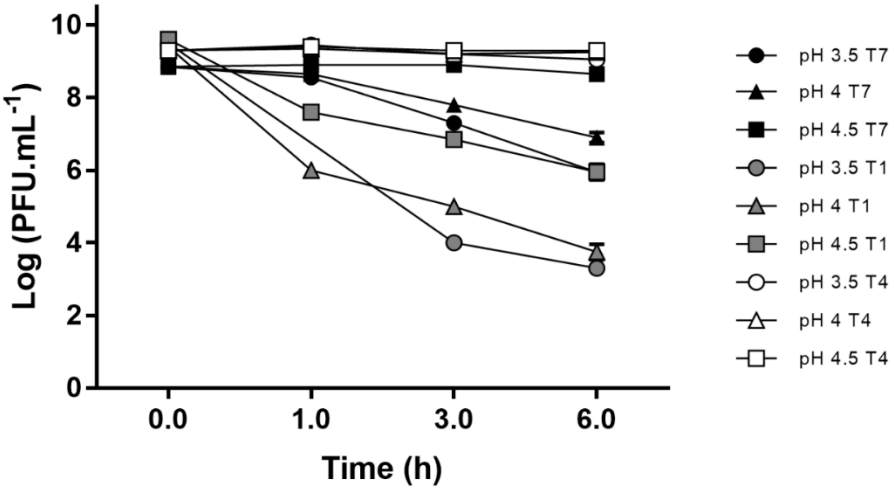

Figure 10: Effect of pH in T7 (dark figures), T1 (grey figures) and T4 (white figure) phage concentration (PFU.mL^{-1}). Data shows each of the three independent assays performed at pH 3.5 (circle), pH 4.0 (triangle) and pH 4.5 (square). LOD = 2 Log; statistical significance, $p<0.05$.

2.3.6 Phage staining in larvae tissue

To confirm phage presence in the larvae tissue, an immunofluorescence assay was conducted. Microscopic observations were performed after incubating samples with a T7 phage antibody conjugated with Alexa Fluor® 488.

Figure 11A shows a stained 10^8 PFU.mL^{-1} T7 phage suspension and allowed to identify strong bright green pixels randomly distributed in the microscopic field (FITC filter). The same bright green signal was observed in tissues from larvae collected 24 hours after they were fed to adult bees (indicated by white arrows in **Figure 11B**). This signal was found in tissues around haemocoel, cavities in between organs whereby the haemolymph circulates (identified in the images as dark circular empty spaces between tissues), on the midgut brush border and with higher intensity in the epithelium of Malpighian tubules (responsible for osmotic and ionic regulation in larvae maintaining homeostasis and excreting toxic and excess substances). The TRITC filter was used in all observations to distinguish the T7 phage-specific bright-green fluorescent signal of Alexa Fluor® 488 from the tissue bright-green autofluorescence.

Some of the details highlighted in the **Figure 11** are present in **Figure 12**, and show that bright green pixels observed in the images obtained with the FITC filter are not present in images from the same microscopic field, captured with the TRITC filter. This is evident in the overlay of FITC and TRITC images, demonstrating that the green dots of the overlapped images are exclusively phages.

Additional observations were performed on samples from the larvae that did not receive phages (negative control). In this case, the image captured by FITC presented the same level of brightness when compared with TRITC filter, revealing the tissue autofluorescence and absence of T7 phage-specific bright-green fluorescent signal of Alexa Fluor® 488. When combining images obtained with both filters no bright-green fluorescent signal was observed (**Figure 13**).

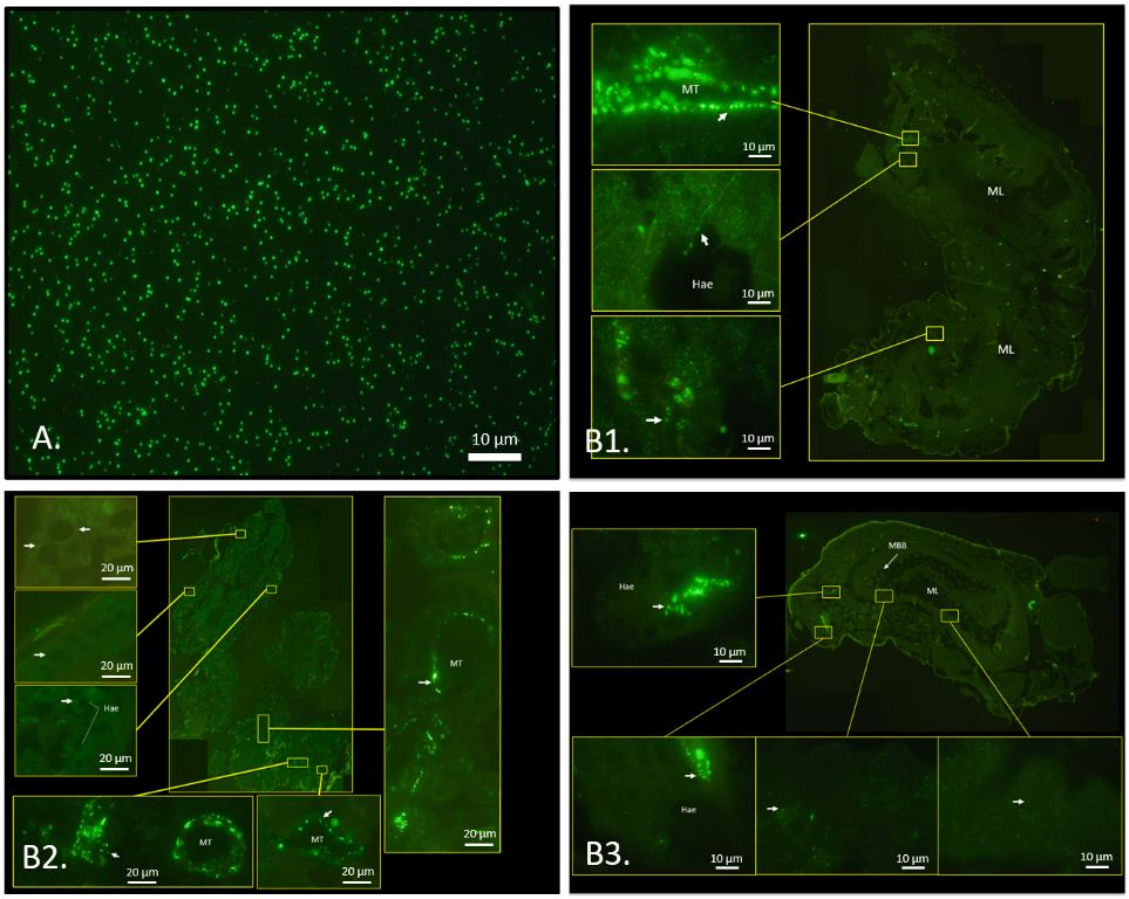

Figure 11: Immunofluorescence staining with T7 tag polyclonal antibody and Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody conjugated with Alexa Fluor® 488. (A) Positive control composed by a 10^8 PFU.mL^{-1} T7 phage suspension. (B) Larvae sections (1st to 3rd instars) for localization of T7 phage (B1: cross section; B2 and B3: longitudinal sections). Each picture shows a global image and emphasizes some details marked with a yellow square. ML: midgut lumen; MBB: midgut brush border; MT: Malpighian tubules; Hae: Haemocoel. Phages are shown as bright green pixels (white arrows) alone or aggregated, depending on the displayed brightness of the dots. Photomicrographs were obtained with 1000× total magnification.

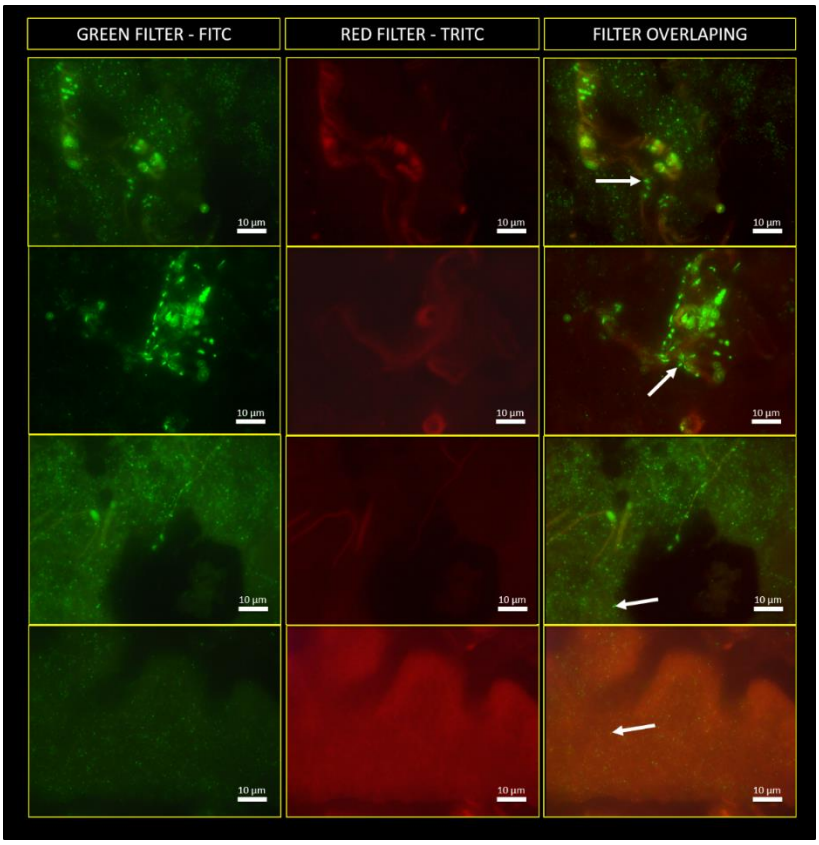

Figure 12: Epifluorescence images showing efficient discrimination between the T7 phage-specific bright-green fluorescent signal of Alexa Fluor® 488 (positive control). Immunofluorescence staining with T7 tag polyclonal antibody and Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody conjugated with Alexa Fluor® 488. The left column show the phage staining (FITC filter); the central column show the tissue autofluorescence (TRITC filter) and the right images present the overlap of the two channels discriminating the T7 phage-specific bright-green fluorescent signal of Alexa Fluor® 488 from the tissue autofluorescence. Arrows indicate phages stained that can easily be visualized on the overlap channel.

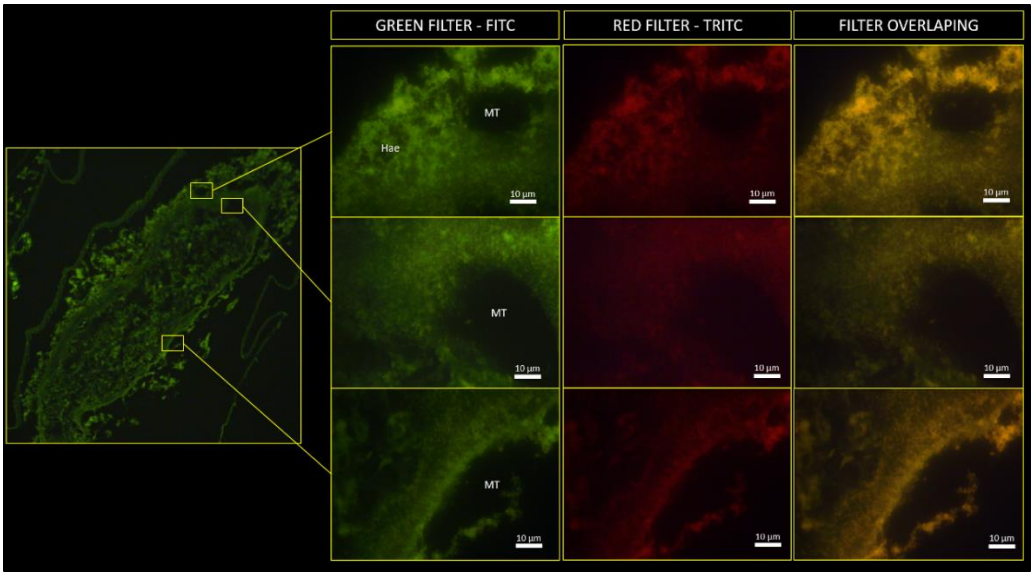

Figure 13: Epifluorescence images showing larvae tissue without phage treatment (negative control). Immunofluorescence staining with T7 tag polyclonal antibody and Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody conjugated with Alexa Fluor® 488 (larvae global image and details with 100× magnitude). The left column show the tissue with no green pixels (FITC filter); the central column show the tissue autofluorescence of tissue (TRITC filter) and the right images present the overlap of the two channels.

2.4 Discussion

The work presented herein plans to clarify phage biodistribution and bioavailability from adult bee to young larvae, particularly when phages are administered orally to adult bees. The purpose was to understand the potential of this delivery strategy to effectively provide viable phage to larvae.

Despite recognizing that spraying hives might be effective in the pathogen control, was consider that the use of phage suspended in adult bees' food may be more advantageous. It seems easier to include it in hive management, it's less time consuming and causes lower product waste.

The phage distribution analysis in adult bees revealed that the amount of phages detected in bee Abd was higher ($p<0.05$) than the obtained in H&T. These differences may be explained not only by the size of gut compartments present in each bee section but also by the influence of peristalsis that rapidly moves the food down the gut.

Phage inactivation or phage efficacy impairment occurring inside the honeybee might be due to hostile conditions that phages have to face after being ingested. In fact, the insect gut poses multiple challenges for microorganisms and viruses ingested with the food. That might include unfavourable pH or harmful digestive enzymes (Douglas, 2015).

T7 phage is known to be stable between pH 6.0 and 8.0 (Kerby & Gowdy, 1949). Most of the bee compartments from the Abd (mid- and hindgut) are acidic – the pHs range from 5.2 to 7.0. The crop (foregut) though stores substances such as nectar and honey whose pH ranges between 4.5 and 5.5 (Anderson et al., 2013; Colibar et al., 2010; Zheng et al., 2017). This acidic effect of the crop may indirectly influence phage stability also in H&T due to trophallaxis. In this action the crop content is regurgitated back to the mouth (Hrncir & Jarau, 2009). We hypothesize that not only pH but also enzymes with proteolytic activity present in bee gut may contribute to phage titre loss. Peptidases such as trypsine and chymotrypsine are present in the midgut where pollen is thought to be broken down. None of these enzymes are found in the crop. This indicates that the proteolysis bias on phages may only occur in Abd (mid- hindgut) (Chan et al., 2013; K. Crailsheim, 1990b).

Phage impairment in H&T might also be due to the presence of RJ, whose antiviral activity had already been reported (Hashemipour, Tavakolineghad, Arabzadeh, Iranmanesh, & Nassab, 2014; Pasupuleti et al., 2017). This substance is produced in the head by the hypopharyngeal glands. The regurgitated crop content is mixed with hypopharyngeal glands' secretions in the first part of the digestive tube, before being deposited in larvae combs. When surrounding larvae RJ was analysed, phages though present lost their infectivity. This drop of viability was confirmed *in vitro*. The T7 phage did not remain infective for more than 3 hours after immersed in commercial RJ (**Figure 9**).

Gochnauer (1970) had already reported phage sensitivity to hive conditions (T. A. Gochnauer, 1970). According to this research phages could not survive if stored in larval food for long periods of time. They needed to be ingested rapidly by larvae in order to reach the nearly neutral alimentary environment (pH 6.8) in which phage might have been stable (Colibar et al., 2010). Yost et al. (2016) also describes inability to recover viable phages from larvae that were artificially fed with RJ mixed only with phage (without host for replication). The negative results reported by Yost et al. (2016) could be due to phage inactivation by RJ before reaching larvae (Yost et al., 2016).

The fact that all phages tested were more tolerant to pH 4.0 than to RJ suggests that the low pH occurring in RJ (3.4–4.5) (Barnutiu et al., 2011) is not the only factor affecting phage stability (Kerby & Gowdy, 1949). The presence of substances such as phenolic compounds (Pasupuleti et al., 2017) and proteinase(s) (Chen, 1995) might contribute to phage inactivation probably through interaction with phage structural proteins (Ali, Alli, Ismail, & Kermasha, 2012).

The type of phage used in the therapy may also influence its behaviour in general hive-derived conditions: T1 and T4 phage survived for longer periods than T7 in RJ (**Figure 9**). This tendency does not seem to be only related to pH sensitivity because T1 was the most susceptible phage at pH 4.0 (**Figure 10**).

Biodistribution assays revealed that phages remain active in adult bee tissues and immunohistochemical analysis confirmed phage transfer from bee to larvae. Microscopic images revealed a wide phage distribution in larvae tissues (**Figure 11A**). After being uptaken by larvae, phages not only reached the midgut lumen but they also penetrated the epithelium. They might have spread then to the haemolymph, as they were found in tissues around the haemocoel. This was confirmed by their presence in Malpighian tubules, which are structures of the excretory system.

However, as discussed above, RJ contains factors that inactivate phages within a 3 hours period. The exposition of phage particles to RJ before being uptaken by larvae might have led to a massive phage inactivation. This accounts for the very low amount of active phage particles present in larvae guts.

Larval midgut conditions (pH around 7.0 (Alvarado et al., 2013) and absence of proteolytic enzymes (K. Crailsheim, 1990b)) are apparently harmless to phages.

Previous reports state that the uptake of around 10 spores are enough to successfully initiate infection in the larvae (Brodsgaard, Ritter, Hansen, & Wolfgang, 1998; Woodrow & Holst, 1942). Nevertheless, this dosage-mortality relationship seems to depend on larval age, genetic constitution and bacterial strain (Genersch, 2006; Genersch et al., 2005). Based on previous reports of exposure bioassays in artificially reared honey bee larvae (Genersch et al., 2005), 100 to 800 colony forming units

(CFU) per mL *P. larvae* cells – about 4 to 40 CFU per larvae – may initiate an infection that will kill 50% of the larvae tested depending on the virulence of the strain. According to the reported infectious doses, the 32 active phage particles would not most likely be sufficient to effectively control the disease.

In conclusion, this work provides evidence that phages administered in bee food are successfully uptaken by the bee and transported in their organs, reaching larvae through the bee-larvae feeding chain. Nevertheless, the amount of viable particles found in larvae seems to be very low to be able to reduce *P. larvae* load and thus to control AFB.

The improvement of the oral delivery effectiveness in the AFB therapeutic might be achieved not only by screening for phages with high tolerance to RJ, but also by the development of strategies that protect phage from general hive-derived conditions or by engineering phages to endure this harsh environment.

References

Abedon, S. T., Kuhl, S. J., Blasdel, B. G., & Kutter, E. M. (2011). Phage treatment of human infections. *Bacteriophage*, *1*(2), 66–85. https://doi.org/10.4161/bact.1.2.15845

Adams, M. H. (1959). *Bacteriophages*. New York: Interscience Publishers.

Ali, H., Alli, I., Ismail, A., & Kermasha, S. (2012). Protein-phenolic interactions in food. *Eurasian Journal of Analytical Chemistry*, *7*(3), 123–133.

Alippi, A. M., López, A. C., Reynaldi, F. J., Grasso, D. H., & Aguilar, O. M. (2007). Evidence for plasmid-mediated tetracycline resistance in Paenibacillus larvae, the causal agent of American foulbrood (AFB) disease in honeybees. *Veterinary Microbiology*, *125*(3–4), 290–303. https://doi.org/10.1016/j.vetmic.2007.05.018

Alvarado, I., Phui, A., Elekonich, M. M., & Abel-Santos, E. (2013). Requirements for in vitro germination of Paenibacillus larvae spores. *Journal of Bacteriology*, *195*(5), 1005–1011. https://doi.org/10.1128/JB.01958-12

Anderson, K. E., Sheehan, T. H., Mott, B. M., Maes, P., Snyder, L., Schwan, M. R., ... Corby-Harris, V. (2013). Microbial ecology of the hive and pollination landscape: Bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (Apis mellifera). *PLoS ONE*, *8*(12), e83125. https://doi.org/10.1371/journal.pone.0083125

Atterbury, R. J. (2009). Bacteriophage biocontrol in animals and meat products, *2*, 601–612. https://doi.org/10.1111/j.1751-7915.2009.00089.x

Barnutiu, L. I., Marghitas, L. Al, Dezmirean, D. S., Mihai, C. M., & Bobis, O. (2011). Chemical composition and antimicrobial activity of royal jelly - Review. *Animal Science and Biotechnologies*, *44*(2), 67–72.

Beims, H., Wittmann, J., Bunk, B., Spröer, C., Rohde, C., Günther, G., ... Steinert, M. (2015). Paenibacillus larvae-Directed bacteriophage HB10c2 and its application in American foulbrood-affected honey bee larvae. *Applied and Environmental Microbiology*, *81*(16), 5411–5419. https://doi.org/10.1128/AEM.00804-15

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of Paenibacillus larvae in honeybee hives. *Journal of Invertebrate Pathology*, *150*(September), 94–100. https://doi.org/10.1016/j.jip.2017.09.010

Brodsgaard, C. J., Ritter, W., Hansen, H., & Wolfgang, C. (1998). Response of in vitro reared honey bee larvae to various doses of Paenibacillus larvae larvae spores. *Apidologie*, *29*, 569–578. https://doi.org/10.1051/apido:19980609

Chan, Q. W. T., Yi Chan, M., Logan, M., Fang, Y., Higo, H., & Foster, L. J. (2013). Honey bee protein atlas at organ-level resolution. *Genome Research*, *23*(11), 1951–1960. https://doi.org/10.1101/gr.155994.113

Chen, C. (1995). Changes in protein components and storage stability of royal jelly under various conditions. *Food Chemistry*, *54*(2), 195–200. https://doi.org/10.1016/0308-8146(95)00031-D

Colibar, O., Popovici, D., Eugeniu, C., & Korodi, G. (2010). The effect of acidifiant on the development of bee families (Apis mellifica). *Med. Vet.*, *43*(2), 296–299.

Crailsheim, K. (1990). The protein balance of the honey bee worker. *Apidologie*, *21*(5), 417–429. https://doi.org/10.1051/apido:19900504

Crailsheim, Karl, & Riessberger-Gallé, U. (2001). Honey bee age-dependent resistance against American foulbrood. *Apidologie*, *32*(1), 91–103. https://doi.org/10.1051/apido:2001114

Djukic, M., Brzuszkiewicz, E., Fünfhaus, A., Voss, J., Gollnow, K., Poppinga, L., ... Daniel, R. (2014). How to kill the honey bee larva: Genomic potential and virulence mechanisms of Paenibacillus larvae. *PLoS ONE*, *9*(3), e90914.

https://doi.org/10.1371/journal.pone.0090914

Douglas, A. E. (2015). Multiorganismal insects: Diversity and function of resident microorganisms. *Annual Review of Entomology*, *60*(1), 17–34. https://doi.org/10.1146/annurev-ento-010814-020822

Drulis-Kawa, Z., Majkowska-Skrobek, G., & Maciejewska, B. (2015). Bacteriophages and phage-derived proteins – Application approaches. *Current Medicinal Chemistry*, *22*(14), 1757–1773. https://doi.org/10.2174/0929867322666150209152851

Genersch, E. (2006). Reclassification of Paenibacillus larvae subsp. pulvifaciens and Paenibacillus larvae subsp. larvae as Paenibacillus larvae without subspecies differentiation. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, *56*(3), 501–511. https://doi.org/10.1099/ijs.0.63928-0

Genersch, E. (2010). American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. *Journal of Invertebrate Pathology*, *103*(SUPPL. 1), S10–S19. https://doi.org/10.1016/j.jip.2009.06.015

Genersch, E., Ashiralieva, A., & Fries, I. (2005). Strain- and Genotype-Specific Differences in Virulence of Paenibacillus larvae subsp. larvae, a Bacterial Pathogen Causing American Foulbrood Disease in Honeybees. *Applied and Environmental Microbiology*, *71*(11), 7551–7555. https://doi.org/10.1128/AEM.71.11.7551-7555.2005

Ghorbani-Nezami, S., LeBlanc, L., Yost, D. G., Amy, P. S., & Jeanne, R. (2015). Phage therapy is effective in protecting honeybee larvae from American foulbrood disease. *Journal of Insect Science*, *15*(1), 1–5. https://doi.org/10.1093/jisesa/iev051

Gochnauer, T. A. (1970). Some properties of a bacteriophage from Bacillus larvae. *Journal of Invertebrate Pathology*, *15*(2), 149–156. https://doi.org/10.1016/0022-2011(70)90228-4

Hansen, H., & Brødsgaard, C. J. (1999). American foulbrood: A review of its biology, diagnosis and control. *Bee World*, *80*(1), 5–23. https://doi.org/10.1080/0005772X.1999.11099415

Hashemipour, M. A., Tavakolineghad, Z., Arabzadeh, S. A. M., Iranmanesh, Z., & Nassab, S. A. H. G. (2014). Antiviral activities of honey, royal jelly, and acyclovir against HSV-1. *Wounds*, *26*(2), 47–54. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-84893923682&partnerID=tZOtx3y1

Hrncir, M., & Jarau, S. (2009). *Food Exploitation by Social Insects - Ecological, Behavioral, and Theoretical Approaches*. *Food Exploitation By Social Insects*. https://doi.org/doi:10.1201/9781420075618.bmatt

Kerby, G. P., & Gowdy, R. a. (1949). Purification pH stability and sedimentation properties of the T7 bacteriophage of Escherichia coli. *Journal of Immunology (Baltimore, Md. : 1950)*, *63*, 93–107.

Lindström, A., Korpela, S., & Fries, I. (2008). The distribution of Paenibacillus larvae spores in adult bees and honey and larval mortality, following the addition of American foulbrood diseased brood or spore-contaminated honey in honey bee (Apis mellifera) colonies. *Journal of Invertebrate Pathology*, *99*(1), 82–86. https://doi.org/10.1016/j.jip.2008.06.010

Oliveira, A., Melo, L. D. R., Kropinski, A. M., & Azeredo, J. (2013). Complete Genome Sequence of the Broad-Host-Range Paenibacillus larvae Phage phiIBB_Pl23. *Genome Announcements*, *1*(5), e00438-13-e00438-13. https://doi.org/10.1128/genomeA.00438-13

Pasupuleti, V. R., Sammugam, L., Ramesh, N., & Gan, S. H. (2017). Honey, propolis, and royal jelly: A comprehensive review of their biological actions and health benefits. *Oxidative Medicine and Cellular Longevity*, *2017*, 1–21. https://doi.org/10.1155/2017/1259510

Rohwer, F., & Edwards, R. (2002). The phage proteomic tree: a genome-based taxonomy for phage. *Journal of Bacteriology*, *184*(16), 4529–4535. https://doi.org/10.1128/JB.184.16.4529-4535.2002

Tian, B., Fadhil, N. H., Powell, J. E., Kwong, W. K., & Moran, N. A. (2012). Long-Term Exposure to Antibiotics Has Caused Accumulation of Resistance Determinants in the Gut Microbiota of Honeybees. *MBio*, *3*(6), 1–7. https://doi.org/10.1128/mBio.00377-12

Woodrow, A. W. (1942). Susceptibility of honeybee larvae to individual inoculations with spores of *Bacillus* larvae. *Journal of Economic Entomology*, *35*(6), 892–895. https://doi.org/10.1093/jee/35.6.892

Woodrow, A. W., & Holst, E. C. (1942). The mechanism of colony resistance to American foulbrood. *Journal of Economic Entomology*, *35*(3), 327–330. https://doi.org/10.1093/jee/35.3.327

Yost, D. G., Tsourkas, P., & Amy, P. S. (2016). Experimental bacteriophage treatment of honeybees (Apis mellifera) infected with Paenibacillus larvae, the causative agent of American foulbrood disease. *Bacteriophage*, *6*(1), e1122698. https://doi.org/10.1080/21597081.2015.1122698

Yue, D., Nordhoff, M., Wieler, L. H., & Genersch, E. (2008). Fluorescence in situ hybridization (FISH) analysis of the interactions between honeybee larvae and Paenibacillus larvae, the causative agent of American foulbrood of honeybees (Apis mellifera). *Environmental Microbiology*, *10*(6), 1612–1620. https://doi.org/10.1111/j.1462-2920.2008.01579.x

Zheng, H., Powell, J. E., Steele, M. I., Dietrich, C., & Moran, N. A. (2017). Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(18), 4775–4780. https://doi.org/10.1073/pnas.1701819114

CHAPTER 3: CHARACTERIZATION OF A NEW PODOVIRUS INFECTING *PAENIBACILLUS LARVAE*

This chapter was based on the following paper:

Ribeiro HG*, Melo LDR, Oliveira H, et al. Characterization of a new podovirus infecting *Paenibacillus larvae*. *Sci Rep*. 2019;9(1):1-12. https://doi.org/10.1038/s41598-019-56699-y

Abstract

The *Paenibacillus larvae* infecting phage API480 (vB_PlaP_API480) is the first reported podovirus for this bacterial species, with an 58 nm icosahedral capsid and a 12 × 8 nm short, non-contractile tail. API480 encodes 77 coding sequences (CDSs) on its 45,026 bp dsDNA genome, of which 47 were confirmed using mass spectrometry. This phage has got very limited genomic and proteomic similarity to any other known ones registered in public databases, including *P. larvae* phages. Comparative genomics indicates API480 is a new species as it is a singleton with 28 unique proteins. Interestingly, the lysis module is highly conserved among *P. larvae* phages, containing a predicted endolysin and two putative holins. The well kept overall genomic organisation (from the structural and morphogenetic modules to the host lysis, DNA replication and metabolism related proteins) confirms a common evolutionary ancestor among *P. larvae* infecting phages. API480 is able to infect 69% of the 61 field strains with an ERIC I genotype, as well as ERIC II strains. Furthermore, this phage is very stable when exposed to high glucose concentrations and to larval gastrointestinal conditions. This highly-specific phage, with its broad lytic activity and stability in hive conditions, might potentially be used in the biocontrol of American Foulbrood disease (AFB).

3.1 Introduction

American foulbrood disease (AFB) is one of the most devastating bacterial diseases affecting honeybees and it is caused by *Paenibacillus larvae*, a Gram-positive worldwide-distributed spore forming bacterium. This infection begins when adult bees provide spore-contaminated food to larvae in the initial stages of development (first 36 hours after egg hatching) causing larvae death (Karl Crailsheim & Riessberger-Gallé, 2001; Genersch, 2010a; Genersch et al., 2005; Woodrow, 1942).

The burning of hives and contaminated material is the compulsory action recommended by authorities to control the proliferation of AFB which causes devastating economic loss to the beekeeping industry and the environment. The use of antibiotics is not advised as they are not active against spores, and they cause further wide-spreading bacterial resistances (Genersch, 2010a; Miyagi et al., 2000). They are also forbidden in Europe (EC regulation 2377/90 and further amendments (European Parliament and the Council of the European Union, 2010)).

Bacteriophages (phages) are now valuable solutions to this infection control. Phages are bacterial viruses that specifically infect their hosts, relying on the cell biosynthetic machinery to produce new viral particles. Phages are considered self-dosing and self-limiting antibacterial agents. After the host lysis, new phages are able to trigger new infection cycles to the surrounding hosts, resulting in exponential phage growth until no host is available (Drulis-Kawa et al., 2015). An important feature of phages is their inactivity in the extracellular environment, thus being innocuous to animals or plants (Atterbury, 2009). The potential of *P. larvae* phages as a tool for treating AFB has been explored by evaluating its efficacy both in infected laboratory-raised larvae (Beims et al., 2015; Ghorbani-Nezami et al., 2015; Yost et al., 2016) and in infected experimental hives (Brady et al., 2017). Up to date, 48 *P. larvae* phage genome sequences have been described. They all belong to the *Siphoviridae* family and they mostly encode known integration genes. Their genomes have been grouped into four clusters (with Fern, Harrison, Vegas and Halcyone as representative phages) and one singleton (phage Lily), based on genomic diversity (Stamereilers et al., 2018). All of these 48 phages seem to have a common evolutionary ancestor, showing an overall common structure.

The isolation and genomic characterization of the first podovirus infecting *P. larvae* is reported here, together with the evaluation of its viability in experimental conditions envisaging the possibility of using this phage in AFB control.

3.2 Material and methods

3.2.1 Bacterial strains: isolation and cultivation conditions

In this study, 23 previously isolated *P. larvae* strains were used: 13 field strains (PI01-(01, 03, 07, 07b2, 13, 14, 18); PI02-(21, 23, 27, 30b, 31); PI03-28) (A. Oliveira et al., 2015); three strains originally isolated in Spain in 2016 (Guadalajara) (PI02-86, 87, 89); seven reference strains: LMG 9820, CCUG 48972, CCUG 48973, LMG 15974, LMG 16252, LMG 16247 and LMG 16250.

These strains were cultivated in MYPGP agar (10 $g.L^{-1}$ Mueller-Hinton Broth (Oxoid); 15 $g.L^{-1}$ yeast extract (Oxoid); 3 $g.L^{-1}$ de K_2HPO_4 (LabKem); 1 $g.L^{-1}$ de Sodium-pyruvate (Fisher); 2% glucose (Ameresco) and 17 $g.L^{-1}$ agar (VWR) and incubated at 37 °C under 5% CO_2 overnight (O/N).

P. larvae isolation was performed as described by Genersch & Otten, (2003), from brood samples collected in 132 hives spread over the Portuguese territory (29 with visible signs of infection and 103 apparently non-infected): larvae were emulsified in 500 µL sterile water, heated at 90 °C, 6 minutes and sewed in MYPGP agar. After incubation for 3 to 6 days at 37 °C, 5% CO_2, single colonies were propagated in MYPGP agar and stored at -80 °C with 20% glycerol.

Non-*P. larvae* strains used to assess phage specificity for *P. larvae* included: *Paenibacillus polymyxa* (LMG 13294), *Paenibacillus alvei* (LMG 13253), *Lactobacillus pentosus* (DSM 20314), *Lactobacillus rhamnosus* (CECT 288), *Lactobacillus paracasei* (CECT 277), *Lactobacillus casei* (CECT 5275), *Lactobacillus acidophilus* (ATCC 4356), *Bacillus subtilis* (DSMZ 10), *Bacillus coagulans* (CECT 12), *Bacillus cereus* (CEB collection), *Bacillus circulans* (CEB collection), *Lactobacillus kunkeei* (LMG 18925) and *Parasaccharibacter apium* alpha 2.2 (strain C6) (Corby-Harris et al., 2014).

All *Lactobacillus* spp were cultured in MRS broth (Frilabo) and MRS 15 $g.L^{-1}$ agar (VWR). *Bacillus* spp, *P. polymyxa* and *P. alvei* were cultured in Nutrient broth ((5 $g.L^{-1}$ Peptone (Amresco) and 3 $g.L^{-1}$ Meat extract (Fluka Biochemika)) and Nutrient agar (15 $g.L^{-1}$ agar). *P. apium* alpha 2.2 was cultured in Sabouraud dextrose broth (SDB) and sewed in SDB 15 $g.L^{-1}$ agar (VWR).

The CCUG strains were obtained from the Culture Collection of the Goteborg University, LMG from the BCCM - Belgian Coordinated Collections of Microorganisms, DSMZ from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and CECT from the Colección Española de Cultivos Tipo.

3.2.2 16S-PCR identification of *P. larvae* and rep-PCR analysis

The bacterial DNA was purified from bacterial suspensions using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo) and amplified using Kapa *Taq* (Kapa Biosystems) according to the manufacturer's instructions.

The PCR primer sequences and conditions used for *P. larvae* identification (**Table 4**) were based on the *P. larvae* 16S rRNA gene (Dobbelaere, de Graaf, & Peeters, 2001). Positive results revealed a 1,106 bp band in a 1% (w/v) agarose gel under UV light.

The Enterobacterial Repetitive Intergenic Consensus (ERIC) genotyping of the previously identified *P. larvae* was accomplished through genomic fingerprinting as reported in Genersch & Otten, (2003) (primers and conditions are detailed in **Table 4**). ERIC patterns were visualised in a 2% agarose gel under UV light. LMG 9820, CCUG 48972, LMG 15974 and LMG 16247 were used as standard for ERIC I, II, III and IV profiles, respectively.

Table 4: PCR conditions for 16S-PCR of *P. larvae* identification and rep-PCR genotyping.

Target gene	Sequence (5′-3′)	PCR conditions
16S rRNA	Fw: CTTGTGTTTCTTTCGGGAGACGCC Rev: TCTTAGAGTGCCCACCTCTGCG	10 minutes 95 °C 15 seconds 95 °C; 15 seconds 60 °C; 15 seconds 72 °C (30 cycles) 3 minutes 72 °C
rep-PCR	ERIC2: AAGTAAGTGACTGGGGTGAGC ERIC1R: ATGTAAGCTCCTGGGGATTCA	3 minutes 95 °C 45 seconds 95 °C; 30 seconds 45 °C; 3 minutes 72 °C (30 cycles) 10 minutes 72 °C

3.2.3 Bacteriophages isolation and production

Soil samples from hive surroundings were used for phage isolation. For that, soil samples were suspended in MYPGP (2x) (1:10 w/v) and then mixed with groups of five different bacterial strains pre-cultured O/N in MYPGP broth (37 °C, 5% CO_2). After another O/N incubation, the supernatant was filtered-sterilized through 0.22 µm PES membranes (GE Healthcare) and 10 µL were spotted on the respective bacterial lawn (bellow designated as "spot test"). For lawns preparation 100 µl of the freshly grown strain was mixed with 3 mL 0.4% MYPGP agar and poured into agar plates. After O/N incubation at 37 °C, 5% CO_2 bacterial inhibition zones were picked and propagated over host bacterial lawns using sterile paper stripes (D. Pires, Sillankorva, Faustino, & Azeredo, 2011). After a subsequent incubation, phages were isolated from a single phage plaque to obtain clones of confluent plaques. A volume of 2 mL SM buffer (5.8 $g.L^{-1}$ NaCl (PanReac); 2 $g.L^{-1}$ $MgSO_4.7H_2O$ (VWR); 50 $mL.L^{-1}$ 1 M Tris-HCl pH 7.5 (VWR)) was added to the plates and afterwards, the floating liquid, together with the soft-agar were collected, centrifuged (10

minutes, 9000 *×g*, 4 °C) and filtered-sterilized through 0.22 µm PES membranes. Phages were stored at 4 °C until use.

For phage propagation, 10 µL of the stored phage suspension were spread evenly on host bacterial lawns (Sillankorva, Neubauer, & Azeredo, 2008). Plates were incubated O/N at 37 °C with 5% CO_2 and treated as described above for phage isolation, until a filter-sterilized high-titre phage suspension around 10^8 PFU.mL^{-1} (PFU: plaque forming unit) was obtained (stored at 4 °C). The diameter of six individual phage plaques was registered using a SZ40 Zoom Stereo Microscope (Olympus).

3.2.4 Lytic spectra determination and efficiency of plating

The lytic activity of the isolated phage vB_PlaP_API480 (API480) was tested against 68 *P. larvae* strains through spot test, as described above for phage isolation. The presence of bacterial inhibition areas was indicative of host susceptibility to the phage, and these strains were further used to assess efficiency of plating (EOP). A volume of 10 µL of serial phage dilutions was placed in each new bacterial lawn and the drop was allowed to drip along the agar surface to facilitate the phage plaque counting. The relative EOP was calculated by dividing the PFU.mL^{-1} of each susceptible strain by the titre for the relevant propagating host (PI02-27) (Melo et al., 2014). The EOP was scored as 0 (negative), 1 (<10%), 2 (10–100%), 3 (>100%) and LFW (Lysis from without) if phage plaques are only visible at the highest dilutions.

3.2.5 Electron microscopy analysis

Phage particles were collected by centrifugation (1 hour, 25000 *×g*, 4 °C) in a Beckman J2-21 centrifuge with a JA18.1 fixed rotor. The sediment was washed twice in tap water prior to centrifugation as above. Phages were deposited on copper grids with a carbon-coated Formvar film grid, stained with 2% uranyl acetate (pH 4.0) and examined using Jeol JEM 1400 transmission electron microscope (Tokyo, Japan).

3.2.6 DNA isolation, genome sequencing and annotation

P. larvae API480 phage genomic DNA was isolated using the phenol-chloroform-isoamyl alcohol method essentially as described in Melo et al., (2014). DNA samples were further used for library construction using the Illumina Nextera XT library preparation kit. The DNA libraries generated were sequenced in the Illumina MiSeq platform, using 250 bp paired-end sequencing reads. An automatic initial treatment was performed on raw sequence data, namely adapters and low-quality bases trimming. Demultiplexed reads were *de novo* assembled into a single contig using Geneious R9 (Biomatters, Newark, NJ, USA).

The assembled genomes were scanned through myRAST to search for coding regions (Aziz et al., 2008) and tRNAscan-SE to search for tRNAs (Schattner, Brooks, & Lowe, 2005). To search for function, proteins were analysed through BLASTp (Altschul, Gish, Miller, Myers, & Lipman, 1990) and HHpred (Soding, Biegert, & Lupas, 2005) using an E-value cut-off of 1×10^{-5} to search for similarities. Identified proteins were also analysed with TMHMM (Käll & Sonnhammer, 2002) and SignalP to predict transmembrane domains and signal peptide cleavage sites (Petersen, Brunak, von Heijne, & Nielsen, 2011). Putative promoter regions were checked using PromoterHunter from phiSITE (Klucar, Stano, & Hajduk, 2010) and were further manually verified. ARNold was used to predict factor-independent terminators (Naville, Ghuillot-Gaudeffroy, Marchais, & Gautheret, 2011) and the energy was calculated using Mfold (Zuker, 2003). The total genome or proteome were checked for antibiotic resistance genes through the ResFinder (Zankari et al., 2012) and the Resistance Gene Identifier (RGI) of CARD (The Comprehensive Antibiotic Resistance Database) (https://card.mcmaster.ca/analyze/rgi). The selected criteria were the display of results with perfect, strict and loose hits. For the search of toxins, the Toxin-Antitoxin Database (TADB) was used with the TAfinder tool (Xie et al., 2018).

3.2.7 Comparative genomic analysis

To determine the relationship of the *P. larvae* API480 podovirus within all *P. larvae* phages, all complete genomes sequences deposited at GenBank as of June 2019 (n=48) were retrieved and analysed as previously described (H. Oliveira et al., 2019). Briefly, the shared gene content was analysed with Phamerator (Cresawn et al., 2011). This program allowed (1) the assignment of all *P. larvae* phage gene products into phams (proteins with related sequences) or orphams (i.e.: unique proteins) using with kclust, an alignment-free algorithm; (2) the generation of pairwise comparison genomic maps; and (3) the identification of conserved domains in all proteins using the NCBI conserved domain database. The resulting protein repertoire relatedness was visualized with SplitsTree (Huson, 1998). Phage membership was assigned based on shared gene content a metric recently used to assigned staphylococcal phages (H. Oliveira et al., 2019), using a cut-off of 40% of shared genes (phams) to assign phages solely in one cluster.

3.2.8 Protein identification by mass spectrometry (ESI-MS/MS)

Mass spectrometry was performed as described in Oliveira et al., (2018). Briefly, phage proteins were extracted from a phage stock, prepared as described above (>10^9 PFU.mL^{-1}) using chloroform-methanol (Acros Organics) (1:1:0.75 in volume). Proteins were subsequently separated by standard SDS-PAGE and stained by Gelcode™ Blue Safe Protein Stain (Thermo Scientific). Bands spanning the entire lane of the gel were cut out and subjected to trypsin digestion. The resulting peptides were then identified by ESI-MS/MS based on a database file containing all predicted phage proteins.

3.2.9 Phage adsorption and One-step growth curve (OSGC)

An O/N grown PI02-27 culture was harvested by centrifugation (10 minutes, 8000 *×g*, 4 °C) and re-suspended in fresh medium to obtain an optical density at 620 nm (OD_{620}) of 0.3 (approximately 3 × 10^7 CFU.mL^{-1}). The phage was added to the obtained suspension of PI02-27 with a multiplicity of infection (MOI) of 0.1 and incubated at 37 °C with shaking (120 rpm in a PSU-10i Orbital Shaker, BIOSAN).

To assess the time the phage takes to adsorb to the host, samples of 50 µL were collected every 10 minutes and for 60 minutes and immediately chloroform-treated (1:10 (v/v)) and centrifuged (2 minutes, 8000 *×g*). The upper phase was serial diluted and the phage was titred (PFU.mL^{-1}) in order to obtain the number of free phages (Sillankorva et al., 2008).

For the OSGC the phage-host adsorption occurred for 35 minutes (as indicated by the adsorption assay) at 37 °C, 120 rpm. The mixture was then centrifuged (10 minutes, 8000 *×g*, 4 °C) and the pellet re-suspended in 10 mL fresh MYPGP broth medium. Samples were taken every 15 minutes over a period of 60 minutes and the phage titre was assessed.

The PFU.$cell^{-1}$ was obtained through the ratio between the PFU.mL^{-1} in each time point and the initial PFU.mL^{-1}. The burst size was estimated from the resultant sigmoid curve.

3.2.10 Phage life cycle

The evaluation of the phage ability to integrate the bacterial host genome was adapted from a procedure suggested by Kalatzis et al., (2017). Briefly, an O/N grown culture of the strain PI02-27 was harvested by centrifugation (10 minutes, 8000 *×g*, 4 °C) and re-suspended in fresh medium to reach an OD_{620} of 0.3. After a tenfold dilution the host suspension was mixed with the phage to get a MOI of 50 and incubated at 37 °C, 5% CO_2 for 24 hours with agitation (120 rpm).

Samples (n=3) were serial diluted and poured in MYPGP agar (incubation at 37 °C, 5% CO_2, O/N). About 10 bacterial colonies were isolated, cultured in solid media and re-cultured again in three serial passages.

The life cycle of API480 was assessed by investigating the presence of the phage into the host genome in each of the isolated colonies. The original PI02-27 strain was used as control. For that, a specific primer pair for API480 was designed based on the CDS_12: 480_12 Fw 5'-CAGGAACTCAGACCCTACGC -3' and 480_12 Rev 5´- GCCTGCTGCAAAGTCATACA -3'. A colony PCR reaction was performed in a MJ Mini Personal Thermal Cycler: 10 minutes at 95 °C followed by 30 cycles of 15 seconds at 95 °C; 15 seconds at 60 °C and 15 seconds at 72 °C and a final extension of 3 minutes at 72 °C. For the reaction mixture a 1x Xpert Fast Master mix (Grisp) and 0.8 µM of each primer were used. A positive reaction revealed a 227 bp band in a 1% agarose gel.

The API480 activity against the obtained colonies was then investigated trough phage spot test in the respective bacterial lawn. Four PI02-H27 bacterial clones newly resistant to the phage (designated as R-PI27) were then tested for phage infection using other *P. larvae* bacteriophages isolated in previous works: phiIBB_PI23 (A. Oliveira et al., 2013); vB_PlaS_CEB16, vB_PI_CEB46, vB_PI_CEB51 and vB_PI_CEB55 (unpublished phages from CEB collection).

3.2.11 Phage specificity and stability in simulated field conditions

The phage specificity for *P. larvae* was evaluated trough spot test on lawns of the above-mentioned non-*P. larvae* bacterial strains including the first instars larval commensal strains *L. kunkeei* and *P. apium* alpha 2.2 (strain C6) (Corby-Harris et al., 2014; Vojvodic et al., 2013).

API480 stability in simulated hive products/solutions was assessed for 24 hours: phage (final concentration of 10^7 PFU.mL^{-1}) was incubated in a 50% (w/v) sucrose solution at room temperature (envisaging phage administration in bees's artificially feed), in royal jelly (RJ) at 37 °C, 5% CO_2 (where first–instars larvae lay on) and in solutions with acidic pH values at 37 °C, 5% CO_2 (that include those occurring in the hive (Anderson et al., 2013; Colibar et al., 2010)).

For the assay with RJ (supplied by Apiguarda, Portugal), API480 was 1:10 (v/v) diluted in 100 µL of this hive-product and incubated for 0, 1, 3, 6, 8 and 24 hours (3 reaction tubes were prepared for each time point). After the addition of 900 µL SM buffer the mixture was homogenised by vortexing and 200 µL of chloroform were added. Tubes were vortexed and centrifuged (2 minutes, 14000 *×g*) and the upper phase was titred (PFU.mL^{-1}).

For the pH assay distinct solutions of universal buffer (UB) (150 mM KCl (PanReac), 10 mM KH_2PO_4 (PanReac), 10 mM Sodium-Citrate (Thermo Fisher Scientific) and 10 mM H_3BO_3 (Thermo Fisher Scientific), were adjusted with HCl (Acros Organics) to obtain a pH range between 3.0 to 5.0 with intervals of 0.5 units. API480 was 1:10 (v/v) diluted and incubated. Two controls were used in this experiment:

phage in SM buffer (pH 7.4) and phage in UB (pH 7.4). Phage concentration ($PFU.mL^{-1}$) was assessed at 0, 1, 3, 6, 8 and 24 hours.

The phage infectivity was still performed in 15 healthy larvae gathered from larvae combs, that were weighted, individually homogenised and incubated with API480 for 24 hours: 100 µL of each homogenate with 10^7 $PFU.mL^{-1}$. After, 900 µL of SM buffer and 200 µL of chloroform were added. Phages were collected from the upper phase of the mixture after centrifugation (2 minutes, 14000 *×g*) and titred ($PFU.mL^{-1}$).

3.2.12 Statistical analysis

The statistical analysis of the results was performed using GraphPad Prism 7. In all the assays, means and standard deviations were determined based on 3 independent experiments (n=3). Results were compared using t-test (phage stability in sucrose 50% (w/v)), one-way ANOVA, with Turkey's multiple comparison statistical test (phage viability on RJ, phage adsorption and OSGC), and two-way ANOVA, with Turkey's multiple comparison statistical test (phage stability with acidic pH and in larvae homogenised). All tests were performed with a confidence level of 95%. Differences were considered statistically different if $p \leq 0.05$.

3.3 Results

3.3.1 Phage isolation and host range

The isolation of new *P. larvae* strains was carried out in order to broaden the geographic and genetic diversity of the collection. A field sample collection carried out throughout 2018 allowed the isolation of 45 strains: 29 from hives with visible signs of infection and 16 from apparently healthy brood. All isolated strains exhibited an identical fingerprint pattern after rep-PCR matching those produced by ERIC I reference strains (**Figure 14**).

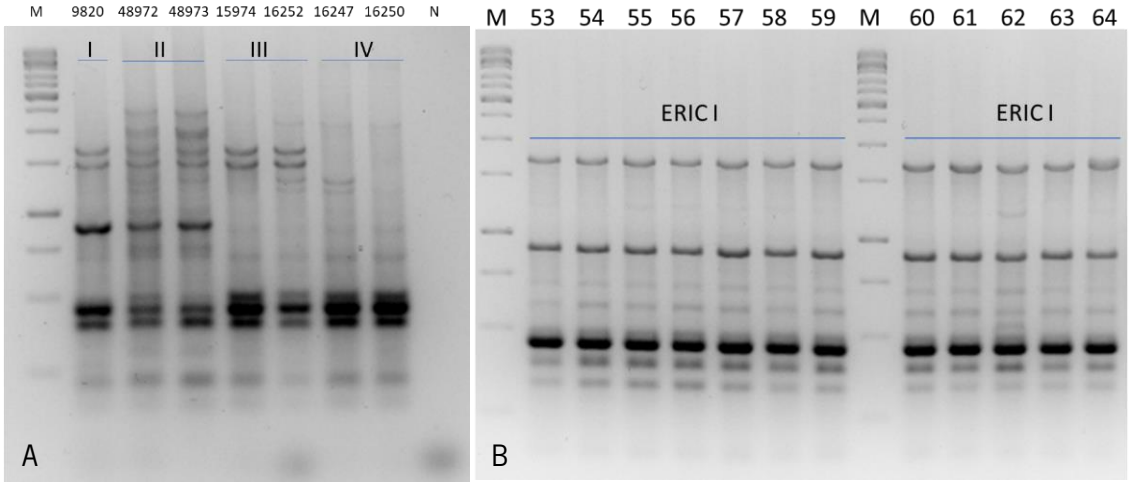

Figure 14: Agarose gel of *P. larvae* genotyping. (A) Standard genotyping using reference strains. (B) Genotype classification of some field strains present in Table 5. M: GeneRuler 1 kb DNA Ladder.

The phage vB_PlaP_API480 (API480) was isolated from a hive soil sample collected in Guadalajara (Spain).

A panel of 68 *P. larvae* strains (including reference strains) were used to evaluate the lytic activity of API480 (**Table 5**) (Supplementary Table S10). API480 revealed a broad lytic spectrum, infecting 69% of the 61 field strains, of which 57% exhibited EOP scores greater than 10%. All remaining strains (31%) were lysed from without. API480 was also able to infect the ERIC II strain CCUG 48972 (EOP <10%) and lysed without replication one of ERIC II, one of ERIC III and two of ERIC IV strains. Only the *P. larvae* strain LMG 16252 (ERIC III) was not lysed by this phage. Additionally, lysis tests in non-*P. larvae* strains revealed that API480 was able to infect *B. circulans* and *B. coagulans*, although those strains do not propagate the phage. All the others were not sensitive to the phage, including the 1st instar larvae commensal strains, *L. kunkeei* and *P. apium* alpha 2.2.

Table 5: API480 lytic spectra and EOP against different strains (*P. larvae* strains were obtained from honey (01), dead larvae (02) and wax (03)). The EOP was scored as 0 (negative), 1 (<10%), 2 (10 – 100%), 3 (>100%) and LFW (Lysis from without). N/A (Non-applicable).

Specie	Strain	Genotype	Score
Paenibacillus larvae	PI01-03; PI02-(23, 30b, 31, 33, 37, 46, 49, 56, 64, 66, 71, 72, 73, 74, 75, 76, 81, 84)	ERIC I	LFW
Paenibacillus larvae	CCUG 48973	ERIC II	LFW
Paenibacillus larvae	LMG 15974	ERIC III	LFW
Paenibacillus larvae	LMG 16247, LMG 16250	ERIC IV	LFW
Paenibacillus larvae	LMG 16252	ERIC III	0
Paenibacillus larvae	PI02-(35, 52, 69, 77, 79, 80, 85)	ERIC I	1
Paenibacillus larvae	CCUG 48972	ERIC II	1
Paenibacillus larvae	PI02-(07, 13, 18, 27, 51, 89)	ERIC I	2
Paenibacillus larvae	LMG 9820	ERIC I	2
Paenibacillus larvae	PI02-(01, 07b2, 14, 21, 34, 36, 45, 47, 48, 50, 53, 54, 55, 57, 58, 59, 60, 61, 62, 63, 65, 67, 68, 70, 78, 83, 86, 87)	ERIC I	3
Paenibacillus larvae	PI03-28	ERIC I	3
Lactobacillus pentosus	DSM 20314	N/A	0
Lactobacillus rhamnosus	CECT 288	N/A	0
Lactobacillus paracasei	CECT 277	N/A	0
Lactobacillus casei	CECT 5275	N/A	0
Lactobacillus acidophilus	ATCC 4356	N/A	0
Lactobacillus kunkeei	LMG 18925	N/A	0
Bacillus subtilis	DSMZ 10	N/A	0
Bacillus cereus	CEB collection	N/A	0
Bacillus circulans	CEB collection	N/A	LFW
Bacillus coagulans	CECT 12	N/A	LFW
Parasaccharibacter apium	Alpha 2.2	N/A	0
Paenibacillus polymyxa	LMG 13294	N/A	0
Paenibacillus alvei	LMG 13253	N/A	0

3.3.2 Phage morphology

API480 forms clear plaques with diameters ranging from 0.9 to 2.6 mm, in 0.4% (w/v) agar plates (**Figure 15A**). Transmission electron microscopy (TEM) images revealed the presence of phage particles with 58 nm diameter icosahedral capsids and 12 × 8 nm short non-contractile tails, belonging to the *Podoviridae* family (**Figure 15B**).

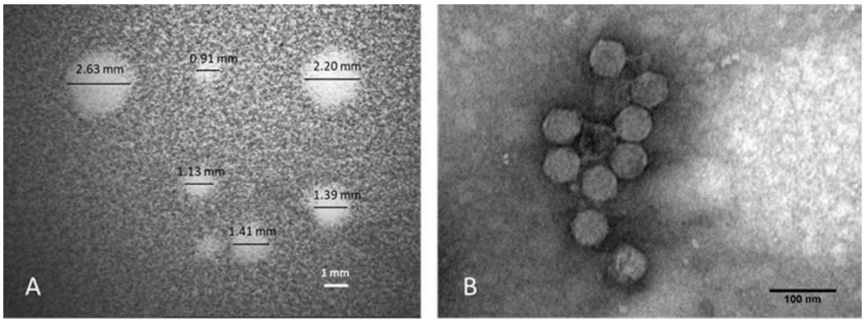

Figure 15: Characteristics of API480. (A) Plaque morphology (black lines indicate the diameter of API480 plaques obtained through a SZ40 Zoom Stereo Microscope (Olympus). Scale bar: 1 mm; (B) Transmission electron micrographs showing the virion particle morphology (stained with 2% uranyl acetate). Scale bar: 100 nm.

3.3.3 Phage genomic and proteomic properties

3.3.3.1 General overview

Phage API480 genome, deposited in the GenBank with the accession number MK533143, is a linear dsDNA molecule of 45,026 bp with 39.24% GC content. API480 encodes 77 coding sequences (CDSs), of which 60 have hypothetical function (being 28 unique to this phage) and only 17 with an assigned function (**Table 6**). Genes are tightly packed achieving 1.71 genes per 1,000 bp, with the genome being 91.9% coded. Furthermore, API480's genome has a translation of 65 proteins that start on ATG codon (84.4%), six on GTG codon (7.8%) and six on TTG codon (7.8%). Although no tRNA or antibiotic resistance genes were identified, ten promoters and eight factor-independent terminators were found, as well as components of the MazEF toxin-antitoxin module, mRNA-degrading endonuclease (gp26) toxin MazF and its antitoxin the MazE (gp27).

The API480 genome is composed by a left-to-right followed by a right-to-left transcription module (**Figure 16**). The DNA packaging and phage morphogenesis genes are located at the beginning of the left arm, similar to the *P. larvae* siphoviruses. Only three proteins with assigned function were identified in this region: terminase large subunit (gp4), portal protein (gp6) and the major capsid protein (gp8). The host lysis proteins are located in the middle of the genome. The endolysin (gp18) is predicted to function as a N-acetylmuramoyl-L-alanine amidase. There are two predicted holins downstream and upstream the endolysin, both with transmembrane domains. Homologs of these putative holin_bhlA (gp17) and the phage_holin_5 (gp21) have been found in most of the *P. larvae* siphoviruses proteins (Stamereilers et al., 2018). The DNA replication, transcription, and metabolism are located in the right arm of the API480 genome: predicted DNA binding protein (gp24), mRNA-degrading endonuclease (gp26), antitoxin MazE (gp27), dUTP pyrophosphatase (gp39), resolvase (gp44), DNA polymerase (gp46), DNA primase (gp47), single-stranded DNA-binding protein (gp48), host nuclease inhibitor protein (gp50), helicase (gp57) and antirestriction protein ArdA (gp70). A similar organization is also observed in the other *P. larvae* phages, for instance, in Wanderer, LincolnB, Harrison and Paisley (**Figure 16**), with the exception of the lysogeny module, which was not identified in the API480 genome.

Table 6: API480 genome annotation and BLASTp or HHpred homology. CDSs unique to API480 are in white; CDSs homologue to bacteria are coloured in light grey; CDSs homologue to phages are coloured in dark grey; proteins with an identified function are highlighted in bold. [1] indicates proteins with assigned function validated by ESI-MS/MS.

gp	Nt Initial	Nt Final	No. Nt	Strand	No. AA	Product	BLASTp/HHpred	E-value
1	1	426	426	+	141	Hypothetical protein	Hypothetical protein [Bacillus thuringiensis]	3E-13
2	404	658	255	+	84	Hypothetical protein		
3	655	963	309	+	102	Hypothetical protein	Hypothetical protein [Bacillus phage Mgbh1]	6E-18
4	956	2407	1452	+	483	**Terminase large subunit** [1]	Phage terminase, large subunit, PBSX family (TIGR01547) [uncultured Mediterranean phage uvMED]	2E-43
5	2420	2611	192	+	63	Hypothetical protein		
6	2627	4405	1779	+	592	**Portal protein** [1]	Portal protein [Enterobacteria phage P22]	1E-46
7	4562	5371	810	+	269	Hypothetical protein	Hypothetical protein [Bacillus cereus]	1E-21
8	5462	6403	942	+	313	**Major capsid protein** [1]	Major capsid protein [Bacillus phage BM5]	1E-39
9	6470	6733	264	+	87	Hypothetical protein	Hypothetical protein [Bacillus thuringiensis]	1E-15
10	6772	7152	381	+	126	Hypothetical protein	Hypothetical protein [Bacillus sp. J37]	2E-06
11	7161	9152	1992	+	663	Hypothetical protein		
12	9164	10342	1179	+	392	Hypothetical protein		
13	10354	10647	294	+	97	Hypothetical protein		
14	10649	11578	930	+	309	Hypothetical protein		
15	11591	12559	969	+	322	Hypothetical protein		
16	12564	17459	4896	+	1631	Hypothetical protein		
17	17841	18083	243	+	80	**Holin** [1]	Holin_BhlA [PF10960.8 hit]	8E-23
18	18101	18772	672	+	223	**Endolysin**	N-acetylmuramoyl-L-alanine amidase [Paenibacillus phage Vegas]	7E-159
19	18778	19248	471	+	156	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	2E-109
20	19266	19541	276	+	91	Hypothetical protein	Hypothetical protein WANDERER_22 [Paenibacillus phage Wanderer]	9E-53
21	19554	19793	240	+	79	**Holin** [1]	Phage_holin_5_1 [PF06946.11 hit]	2E-17
22	19881	20150	270	-	89	Hypothetical protein		
23	20190	20432	243	-	80	Hypothetical protein	Nucleotide binding domain [Escherichia coli K12]	1E-12
24	20425	21408	984	-	327	**DNA-binding protein** [1]	ParM/StbA family protein [Bacillus mycoides]	3E-59
25	21603	21893	291	-	96	Hypothetical membrane protein	Hypothetical protein [Paenibacillus larvae]	7E-47

26	21920	22249	330	-	109	**mRNA-degrading endonucleas** [1]	mRNA-degrading endonuclease [Paenibacillus larvae]	2E-73
27	22246	22515	270	-	89	**Antitoxin MazE**	Antitoxin MazE [Paenibacillus larvae subsp. larvae]	3E-54
28	22580	22777	198	+	65	Hypothetical membrane protein		
29	22906	23064	159	-	52	Hypothetical protein		
30	23057	23431	375	-	124	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	6E-75
31	23415	23603	189	-	62	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	4E-31
32	23600	23800	201	-	66	Hypothetical membrane protein		
33	23805	23996	192	-	63	Hypothetical membrane protein		
34	23981	24241	261	-	86	Hypothetical protein	Hypothetical protein TRIPP_91 [Paenibacillus phage Tripp]	2E-37
35	24243	24428	186	-	61	Hypothetical membrane protein		
36	24484	24864	381	-	126	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	3E-71
37	24871	25161	291	-	96	Hypothetical protein	Hypothetical protein WANDERER_61 [Paenibacillus phage Wanderer]	8E-60
38	25154	25477	324	-	107	Hypothetical protein	Hypothetical protein C7CDELTA_68 [Paenibacillus phage C7Cdelta]	6E-58
39	25507	26010	504	-	167	**dUTP pyrophosphatase** [1]	dUTP pyrophosphatase [Paenibacillus phage C7Cdelta]	9E-72
40	26022	26282	261	-	86	Hypothetical protein	Hypothetical protein VEGAS_60 [Paenibacillus phage Vegas]	7E-12
41	26298	26417	120	-	39	Hypothetical protein	Hypothetical protein VEGAS_59 [Paenibacillus phage Vegas]	3E-20
42	26414	26602	189	-	62	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	1E-22
43	26605	26805	201	-	66	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	4E-34
44	26798	27301	504	-	167	**Resolvase** [1]	Holliday junction resolvase [Escherichia coli]	2,80E-24
45	27303	27497	195	-	64	Hypothetical protein		
46	27613	29838	2226	-	741	**DNA polymerase I** [1]	DNA polymerase I [Geobacillus stearothermophilus]	3E-76
47	29870	32662	2793	-	930	**DNA primase** [1]	DNA primase small subunit [Bacillus phage vB_BboS-125]	2E-12
48	32686	33147	462	-	153	**Single-stranded DNA-binding protein** [1]	Single-stranded DNA-binding protein [Paenibacillus larvae]	9E-86
49	33214	33756	543	-	180	Hypothetical protein		
50	33807	34355	549	-	182	**Host-nuclease inhibitor** [1]	Host-nuclease inhibitor [Desulfovibrio vulgaris]	3E-28
51	34336	34542	207	-	68	Hypothetical protein	Hypothetical protein WANDERER_58 [Paenibacillus phage Wanderer]	3E-17
52	34532	34774	243	-	80	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	1E-49

53	34767	34997	231	-	76	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	3E-38
54	35004	35312	309	-	107	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	6E-69
55	35359	35541	183	-	60	Hypothetical protein	Hypothetical protein WANDERER_53 [Paenibacillus phage Wanderer]	7E-26
56	35538	35798	261	-	86	Hypothetical protein		
57	35802	37370	1569	-	522	**DEAD/DEAH box helicase** [1]	DEAD/DEAH box helicase [Bacillus thuringiensis]	3E-129
58	37363	37515	153	-	50	Hypothetical protein		
59	37796	38482	687	-	228	Hypothetical protein	Hypothetical protein [Bacillus cereus]	8E-44
60	38495	39013	519	-	172	Hypothetical protein	Hypothetical protein [Bacillus cereus]	2E-20
61	39031	39198	168	-	55	Hypothetical membrane protein		
62	39188	39346	159	-	52	Hypothetical protein		
63	39387	39638	252	-	83	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	2E-43
64	39673	39876	204	-	67	Hypothetical protein		
65	39889	40290	402	-	133	Hypothetical protein		
66	40355	40981	627	-	208	Hypothetical protein		
67	40968	41144	177	-	58	Hypothetical membrane protein		
68	41189	41380	192	-	63	Hypothetical protein		
69	41402	41584	183	-	60	Hypothetical protein		
70	41614	42114	501	-	166	**Antirestriction protein ArdA** [1]	Antirestriction protein ArdA [Paenibacillus larvae]	6E-26
71	42165	42347	183	-	60	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	5E-24
72	42358	42618	261	-	86	Hypothetical protein		
73	42742	42987	246	-	81	Hypothetical protein	Hypothetical protein [Brevibacillus laterosporus]	1E-22
74	42992	43219	228	-	75	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	1E-36
75	43546	43677	132	-	43	Hypothetical protein		
76	43704	44159	456	-	151	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	1E-32
77	44461	44676	216	-	71	Hypothetical protein	Hypothetical protein JENST_60 [Brevibacillus phage Jenst]	5E-07

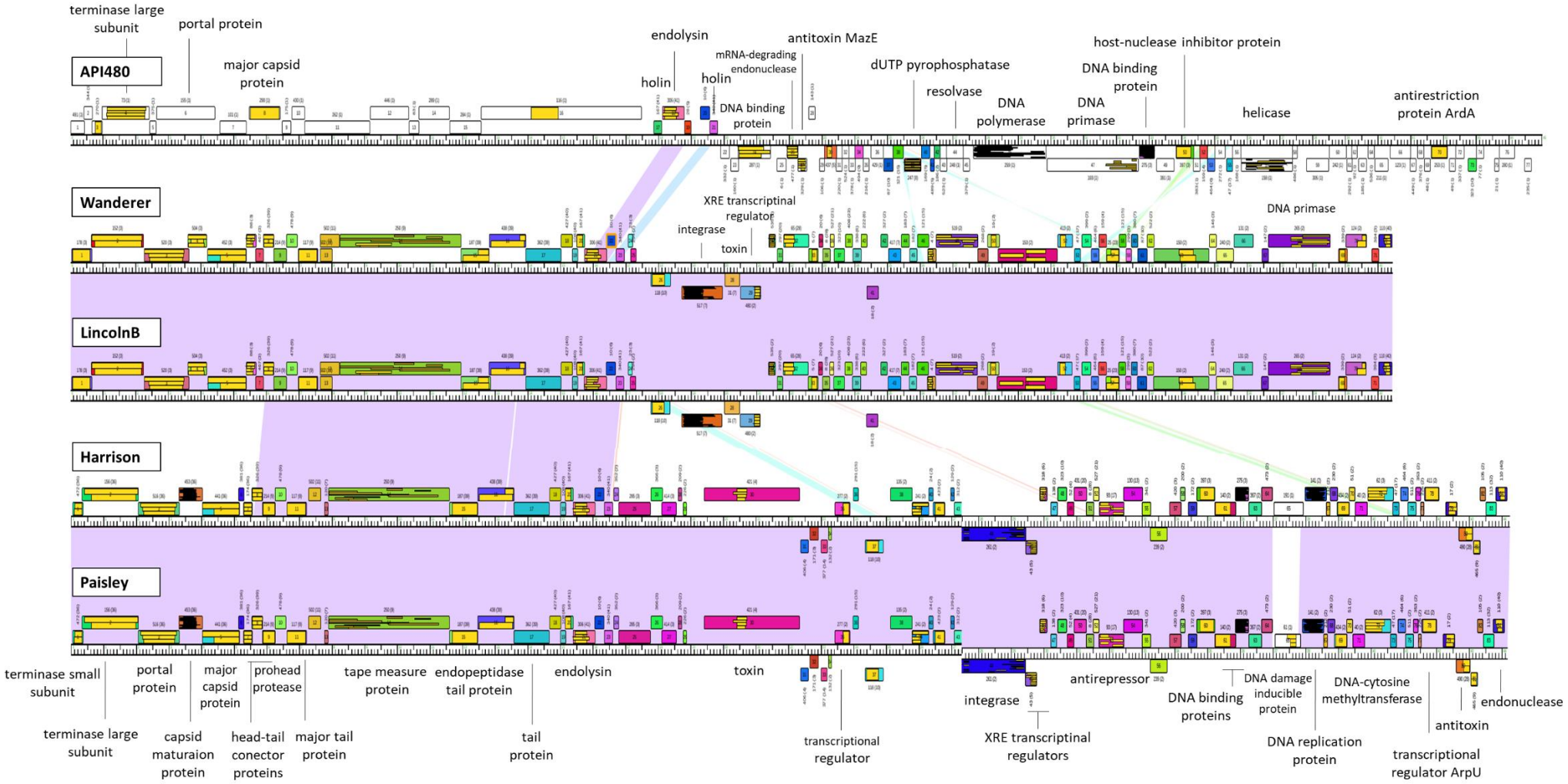

Figure 16: Pairwise genome maps. API480 whole-genome was compared with the closest relatives, Wanderer, LincolnB, Harrison and Paisley. Maps were created with Phamerator. Pairwise sequence similarity (minimal BLASTn cut-off E-value is 10^{-5}) is indicated according to colour spectrum where purple and red lines denote regions of highest and lowest nucleotide similarity, respectively. Gene products are labelled with predicted function (phams i.e. proteins members have the same colour, orphams i.e. unique proteins are shown in white). Their positioning either above or below the bar correspond to rightwards or leftwards transcription, respectively.

3.3.3.2 Comparative analysis

An initial API480 genome alignment by BLASTn revealed that API480 genome is distinct from other available genomes in the GenBank. The highest genome coverage obtained to an E-value of 0.0 was only 5% by LincolnB and Wanderer with an identity of 85.6% and 89.3%, respectively. As mentioned previously, the levels of amino acid identity of API480-encoding proteins are very limited and when existent they are mostly found against *P. larvae* phage proteins (<55% average amino acid identity), indicating the lack of a close relationship between API480 and any phage currently known.

To improve the placement of the API480 genome in the context of the *P. larvae* phage population, we compared all *P. larvae* phages through shared gene content. Comparative analysis of all 49 *P. larvae* phage genomes resulted in the establishment of a new singleton enclosing API480 among four clusters (Fern, Harrison, Vegas and Halcyone) and one singleton (Lily), previously described by Stamereilers et al. (2018) (Stamereilers et al., 2018). The four clusters and the two singletons are shown on **Figure 17** and their relationship was represented by the branch lengths. This clustering joins phages that share more than 40% of their proteins with all members of the same cluster (**Table 7**). While Lily still shares a maximum of 39% of its proteins with some phages (Arcticfreeze, Devri, Bloom, Jacopo, Genki and Gryphonian), API480 is an even more distant phage as its only shares 14% or fewer proteins. Wanderer (13.6%), LincolnB (13.6%), Harrison (11.2%) and Paisley (11.2%) can be considered as the closest relatives (**Table 7**).

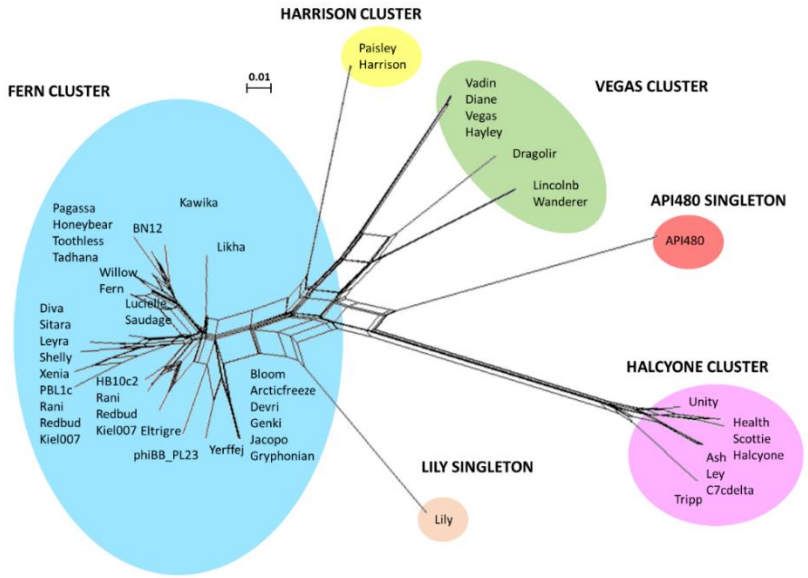

Figure 17: Diversity of *P. larvae* phages genomes. A total of 49 *P. larvae* phages (48 siphoviruses and 1 podovirus – API480) were compared with Phamerator in 3D and the relationship of shared gene content was visualized into 2D space with Splitstree. Clusters assigned based on sharing >40% gene products are highlighted with colours.

Through the Phamerator analysis it was also possible to see that only 19 out of the 77 predicted API480 proteins are shared with *P. larvae* siphoviruses (**Table 8**) and that Harrison and Vegas are the most closely related clusters to the API480. While no universal proteins were found, it is clear that the host lysis proteins (holin_bhlA, endolysin and the holin) are mostly conserved. This is evidenced by the presence of these proteins in all phages from different clusters and singletons, except for Halcyone cluster (**Table 8**). It is also visible in the Phamerator genome map that the only area highlighted in purple shading between genomes (nucleotide sequence most similar), refers to the endolysin site (**Figure 16**).

Table 7: Shared gene content. The complete genome sequences of all *P. larvae* phages (n=49) was comparative analysed through Phamerator and the resulting shared gene content matrix was plotted. Phamerator have assigned gene products into phams (proteins with related sequences) or orphams (i.e. unique proteins). Phage groups are defined into cluster (phage groups) on the basis of sharing >40% of their proteins with all members or as or singletons (single phages) if sharing fewer proteins. In the upper part, the cluster division is highlighted with colours and match the previous classification here (API480 in red). Phages that share >40% of their gene products are coloured in grey.

	FERN																											HARRISON		VEGAS							LILY	HALCYONE								API140
PAGASSA	100	97.1	95.7	93.4	83.8	83.8	79.8	79.8	88	87	68.9	70.1	70.1	72.6	67	61.2	61.2	61.2	61.2	61.2	61.2	69.3	60.8	62.8	66.2	73.9	66.2	62.6	61.6	61.5	29	29	31.3	31.3	31.3	31.7	17.5	21.6	21.6	28	1.3	1.3	1.3	2.6	2.6	2.6	1.4	2.6	4.1
HONEYBEAR	97.1	100	98.5	89.9	83.4	83.4	82.2	82.2	86.4	89.6	70.9	72.1	72.1	74.8	68.8	63.1	63.1	63.1	63.1	63.1	63.1	71.3	62.6	64.7	68.3	76	68.2	63.2	62.1	62.1	30	30	32.4	32.4	32.4	32.7	16.5	22.3	22.3	28.9	1.4	1.4	1.4	2.7	2.7	2.7	1.4	2.6	4.3
TOOTHLESS	95.7	98.5	100	91.3	84.7	84.7	83.5	83.5	87.8	91.1	72	73.2	73.2	76	69.8	64.1	64.1	64.1	64.1	64.1	64.1	72.4	63.6	65.7	69.5	77.1	69.3	64.3	63.2	63.1	30.6	30.6	30.3	30.3	30.3	30.6	16.8	22.6	22.6	29.4	1.4	1.4	1.4	2.7	2.7	2.7	1.5	2.7	4.3
TADHANA	93.4	89.9	91.3	100	90.1	90.1	81.2	81.2	84.1	82.8	69.9	71	71	67.7	69.3	65.1	65.1	65.1	65.1	65.1	65.1	75	66.2	68.2	67.4	76.5	67.2	63.7	62.6	62.6	33	33	30	30	30	30.3	18.2	22.4	22.4	30.6	2.7	2.7	2.7	4	4	4	2.9	4	4.3
FERN	83.8	83.4	84.7	90.1	100	100	90.1	90.1	74.9	83.8	63.7	64.8	64.8	72.2	64.6	60.6	60.6	60.6	60.6	60.6	60.6	70.3	70.7	72.6	61.5	70.1	62.7	60.8	58.3	69.4	32.2	32.2	30.5	30.5	30.5	30.9	19.3	23.4	23.4	29.9	5.3	5.3	5.3	3.9	3.9	3.9	2.8	3.9	5.6
WILLOW	83.8	83.4	84.7	90.1	100	100	90.1	90.1	74.9	83.8	63.7	64.8	64.8	72.2	64.6	60.6	60.6	60.6	60.6	60.6	60.6	70.3	70.7	72.6	61.5	70.1	62.7	60.8	58.3	69.4	32.2	32.2	30.5	30.5	30.5	30.9	19.3	23.4	23.4	29.9	5.3	5.3	5.3	3.9	3.9	3.9	2.8	3.9	5.6
LUCIELLE	79.8	82.2	83.5	81.2	90.1	90.1	100	100	72.3	82.8	73	74.3	74.3	70.8	67.6	60.5	60.5	60.5	60.5	60.5	60.5	64.1	67.7	66.7	70.4	71.7	71.8	68.1	65.6	76.8	26.2	26.2	30	30	30	30.3	16.7	22.4	22.4	29.1	4.1	4.1	4.1	2.7	2.7	2.7	1.4	2.7	5.7
SAUDAGE	79.8	82.2	83.5	81.2	90.1	90.1	100	100	72.3	82.8	73	74.3	74.3	70.8	67.6	60.5	60.5	60.5	60.5	60.5	60.5	64.1	67.7	66.7	70.4	71.7	71.8	68.1	65.6	76.8	26.2	26.2	30	30	30	30.3	16.7	22.4	22.4	29.1	4.1	4.1	4.1	2.7	2.7	2.7	1.4	2.7	5.7
BN12	88	86.4	87.8	84.1	74.9	74.9	72.3	72.3	100	79.5	67.5	68.8	68.8	71.1	65.7	60	60	60	60	60	60	64.9	59.5	61.5	62.1	69.4	62	57.2	57.5	56.1	28.4	28.4	29.3	29.3	29.3	27.1	15.7	21.1	21.1	28.8	1.3	1.3	1.3	5.1	5.1	5	1.4	2.5	4
KAWIKA	87	89.6	91.1	82.8	83.8	83.8	82.8	82.8	79.5	100	64.3	65.4	65.4	74.1	63.7	59.8	59.8	59.8	59.8	59.8	59.8	67.8	62.3	64.2	63.4	69.2	64.7	61.2	58.8	61.5	30.4	30.4	31.3	31.3	31.3	31.7	17.5	28.8	28.8	29.4	2.6	2.6	2.6	2.6	2.6	2.6	1.4	2.6	8.2
KIEL007	68.9	70.9	72	69.9	63.7	63.7	73	73	67.5	64.3	100	98.4	98.4	84.5	89.2	66.2	66.2	66.2	66.2	66.2	66.2	66.7	62.6	63.2	82	72.8	71.4	66.3	81.5	68	28	28	26.4	26.4	26.4	26.6	17	21.3	21.3	29.6	4.2	4.2	4.2	2.7	2.7	2.7	1.5	4.1	4.4
REDBUD	70.1	72.1	73.2	71	64.8	64.8	74.3	74.3	68.8	65.4	98.4	100	100	84.3	90.6	67.3	67.3	67.3	67.3	67.3	67.3	67.8	63.6	64.3	83.5	74	72.7	67.5	80	67.8	28.6	28.6	26.9	26.9	26.9	27.1	17.3	21.7	21.7	30.2	4.2	4.2	4.3	2.8	2.8	2.8	1.5	4.2	4.4
RANI	70.1	72.1	73.2	71	64.8	64.8	74.3	74.3	68.8	65.4	98.4	100	100	84.3	90.6	67.3	67.3	67.3	67.3	67.3	67.3	67.8	63.6	64.3	83.5	74	72.7	67.5	80	67.8	28.6	28.6	26.9	26.9	26.9	27.1	17.3	21.7	21.7	30.2	4.2	4.2	4.3	2.8	2.8	2.8	1.5	4.2	4.4
ELTIGRE	72.6	74.8	76	67.7	72.2	72.2	70.8	70.8	71.1	74.1	84.5	84.3	84.3	100	81.7	61.1	61.1	61.1	61.1	61.1	61.1	66.2	69.7	73.2	69.2	65.8	60.2	57	68.8	64.3	32.5	32.5	28.1	28.1	28.1	28.4	17.9	22.1	22.1	28.7	5.4	5.4	5.4	2.7	2.7	2.6	1.4	3.9	4.2
HB10C2	67	68.8	69.8	69.3	64.6	64.6	67.6	67.6	65.7	63.7	89.2	90.6	90.6	81.7	100	60.4	60.4	60.4	60.4	60.4	60.4	65.9	61.7	64.1	76.5	70.3	64.6	61.5	72.9	60.5	31.6	31.6	28.3	28.3	28.3	28.6	18.1	22.7	22.7	28.5	4.5	4.5	4.5	3	3	2.9	1.6	4.4	4.7
ARCTICFREEZE	61.2	63.1	64.1	65.1	60.6	60.6	60.5	60.5	60	59.8	66.2	67.3	67.3	61.1	60.4	100	98.5	98.5	98.5	98.5	98.5	66.7	62.6	75.2	61.1	63.1	63.7	58.9	62.1	55	32.7	32.7	28.4	28.4	28.4	28.7	27.1	19.3	19.3	39	2.7	2.7	2.7	2.7	2.7	2.7	1.4	1.3	5.7
DEVRI	61.2	63.1	64.1	65.1	60.6	60.6	60.5	60.5	60	59.8	66.2	67.3	67.3	61.1	60.4	98.5	100	98.5	98.5	98.5	98.5	66.7	62.6	75.2	61.1	63.1	63.7	58.9	62.1	55	32.7	32.7	28.4	28.4	28.4	28.7	27.1	19.3	19.3	39	2.7	2.7	2.7	2.7	2.7	2.7	1.4	1.3	5.7
BLOOM	61.2	63.1	64.1	65.1	60.6	60.6	60.5	60.5	60	59.8	66.2	67.3	67.3	61.1	60.4	98.5	98.5	100	98.5	98.5	98.5	66.7	62.6	75.2	61.1	63.1	63.7	58.9	62.1	55	32.7	32.7	28.4	28.4	28.4	28.7	27.1	19.3	19.3	39	2.7	2.7	2.7	2.7	2.7	2.7	1.4	1.3	5.7
JACOPO	61.2	63.1	64.1	65.1	60.6	60.6	60.5	60.5	60	59.8	66.2	67.3	67.3	61.1	60.4	98.5	98.5	98.5	100	98.5	98.5	66.7	62.6	75.2	61.1	63.1	63.7	58.9	62.1	55	32.7	32.7	28.4	28.4	28.4	28.7	27.1	19.3	19.3	39	2.7	2.7	2.7	2.7	2.7	2.7	1.4	1.3	5.7
GENKI	61.2	63.1	64.1	65.1	60.6	60.6	60.5	60.5	60	59.8	66.2	67.3	67.3	61.1	60.4	98.5	98.5	98.5	98.5	100	98.5	66.7	62.6	75.2	61.1	63.1	63.7	58.9	62.1	55	32.7	32.7	28.4	28.4	28.4	28.7	27.1	19.3	19.3	39	2.7	2.7	2.7	2.7	2.7	2.7	1.4	1.3	5.7
GRYPHONIAN	61.2	63.1	64.1	65.1	60.6	60.6	60.5	60.5	60	59.8	66.2	67.3	67.3	61.1	60.4	98.5	98.5	98.5	98.5	98.5	100	66.7	62.6	75.2	61.1	63.1	63.7	58.9	62.1	55	32.7	32.7	28.4	28.4	28.4	28.7	27.1	19.3	19.3	39	2.7	2.7	2.7	2.7	2.7	2.7	1.4	1.3	5.7
LIKHA	69.3	71.3	72.4	75	70.3	70.3	64.1	64.1	64.9	67.8	66.7	67.8	67.8	66.2	65.9	66.7	66.7	66.7	66.7	66.7	66.7	100	64.6	65.2	63	68.4	71.8	68.1	69.9	62.6	37.2	37.2	30	30	30	30.3	16.7	22.4	22.4	36.4	4.1	4.1	4.1	4	4	4	2.9	6.7	5.7
PHIIBB_PL23	60.8	62.6	63.6	66.2	70.7	70.7	67.7	67.7	59.5	62.3	62.6	63.6	63.6	69.7	61.7	62.6	62.6	62.6	62.6	62.6	62.6	64.6	100	74.6	57.7	62.6	58.6	55.5	55.9	60.1	43.3	43.3	28.1	28.1	28.1	28.4	16.4	22.1	22.1	31.5	4	4	4	2.7	2.7	2.6	2.8	5.3	7
YERFFEJ	62.8	64.7	65.7	68.2	72.6	72.6	66.7	66.7	61.5	64.2	63.2	64.3	64.3	73.2	64.1	75.2	75.2	75.2	75.2	75.2	75.2	65.2	74.6	100	58.2	63.3	56.3	53.3	55	57.8	39.9	39.9	29	29	29	29.3	22.1	18.8	18.8	31	5.3	5.3	5.3	3.9	3.9	3.9	2.8	3.9	4.2
SITARA	66.2	68.3	69.5	67.4	61.5	61.5	70.4	70.4	62.1	63.4	82	83.5	83.5	69.2	76.5	61.1	61.1	61.1	61.1	61.1	61.1	63	57.7	58.2	100	85.8	84.4	79.7	84.4	74.3	24.3	24.3	29.1	29.1	29.1	29.4	17	18.2	18.2	28.6	5.1	5.1	5.1	3.8	3.8	3.7	1.3	5	4
DIVA	73.9	76	77.1	76.5	70.1	70.1	71.7	71.7	69.4	69.2	72.8	74	74	65.8	70.3	63.1	63.1	63.1	63.1	63.1	63.1	68.4	62.6	63.3	85.8	100	89.2	83.3	76.2	77.4	27.4	27.4	30	30	30	30.3	17.4	20.3	20.3	32	1.4	1.4	1.4	1.4	1.4	1.4	1.5	2.8	4.5
SHELLY	66.2	68.2	69.3	67.2	62.7	62.7	71.8	71.8	62	64.7	71.4	72.7	72.7	60.2	64.6	63.7	63.7	63.7	63.7	63.7	63.7	71.8	58.6	56.3	84.4	89.2	100	93.3	85.3	84.6	28.2	28.2	30.5	30.5	30.5	30.9	16.3	20.4	20.4	37	2.7	2.7	2.7	2.6	2.6	2.6	1.4	5.2	5.6
XENIA	62.6	63.2	64.3	63.7	60.8	60.8	68.1	68.1	57.2	61.2	66.3	67.5	67.5	57	61.5	58.9	58.9	58.9	58.9	58.9	58.9	68.1	55.5	53.3	79.7	83.3	93.3	100	84.6	85	26.5	26.5	28.7	28.7	28.7	29	16.8	19.3	19.3	34.9	2.5	2.5	2.5	2.5	2.5	2.5	1.3	4.9	5.3
LEYRA	61.6	62.1	63.2	62.6	58.3	58.3	65.6	65.6	57.5	58.8	81.5	80	80	68.8	72.9	62.1	62.1	62.1	62.1	62.1	62.1	69.9	55.9	55	84.4	76.2	85.3	84.6	100	73.7	26.7	26.7	27.7	27.7	27.7	28	18.3	18.1	18.1	32.4	5	5	5.1	3.7	3.7	3.7	1.3	6.2	5.3
PBL1C	61.5	62.1	63.1	62.6	69.4	69.4	76.8	76.8	56.1	61.5	68	67.8	67.8	64.3	60.5	55	55	55	55	55	55	62.6	60.1	57.8	74.3	77.4	84.6	85	73.7	100	24.7	24.7	29.3	29.3	29.3	29.7	17.9	20.3	20.3	34.2	4.9	4.9	4.9	2.4	2.4	2.4	1.3	4.8	5.2
HARRISON	29	30	30.6	33	32.2	32.2	26.2	26.2	28.4	30.4	28	28.6	28.6	32.5	31.6	32.7	32.7	32.7	32.7	32.7	32.7	37.2	43.3	39.9	24.3	27.4	28.2	26.5	26.7	24.7	100	98.8	34.1	34.1	34.1	34.5	25.3	28.8	28.8	17.8	1.2	1.2	1.2	1.2	1.2	1.2	1.3	4.6	11.2
PAISLEY	29	30	30.6	33	32.2	32.2	26.2	26.2	28.4	30.4	28	28.6	28.6	32.5	31.6	32.7	32.7	32.7	32.7	32.7	32.7	37.2	43.3	39.9	24.3	27.4	28.2	26.5	26.7	24.7	98.8	100	34.1	34.1	34.1	34.5	25.3	28.8	28.8	17.8	1.2	1.2	1.2	1.2	1.2	1.2	1.3	4.6	11.2
DIANE	31.3	32.4	30.3	30	30.5	30.5	30	30	29.3	31.3	26.4	26.9	26.9	28.1	28.3	28.4	28.4	28.4	28.4	28.4	28.4	30	28.1	29	29.1	30	30.5	28.7	27.7	29.3	34.1	34.1	100	100	100	98.8	50	41.5	41.5	12.6	2.3	2.3	2.3	3.4	3.4	2.3	0	2.3	9.8
VADIM	31.3	32.4	30.3	30	30.5	30.5	30	30	29.3	31.3	26.4	26.9	26.9	28.1	28.3	28.4	28.4	28.4	28.4	28.4	28.4	30	28.1	29	29.1	30	30.5	28.7	27.7	29.3	34.1	34.1	100	100	100	98.8	50	41.5	41.5	12.6	2.3	2.3	2.3	3.4	3.4	2.3	0	2.3	9.8
VEGAS	31.3	32.4	30.3	30	30.5	30.5	30	30	29.3	31.3	26.4	26.9	26.9	28.1	28.3	28.4	28.4	28.4	28.4	28.4	28.4	30	28.1	29	29.1	30	30.5	28.7	27.7	29.3	34.1	34.1	100	100	100	98.8	50	41.5	41.5	12.6	2.3	2.3	2.3	3.4	3.4	2.3	0	2.3	9.8
HAYLEY	31.7	32.7	30.6	30.3	30.9	30.9	30.3	30.3	27.1	31.7	26.6	27.1	27.1	28.4	28.6	28.7	28.7	28.7	28.7	28.7	28.7	30.3	28.4	29.3	29.4	30.3	30.9	29	28	29.7	34.5	34.5	98.8	98.8	98.8	100	50.6	41.9	41.9	12.7	2.4	2.4	2.4	3.5	3.5	2.3	0	2.3	10
DRAGOLIR	17.5	16.5	16.8	18.2	19.3	19.3	16.7	16.7	15.7	17.5	17	17.3	17.3	17.9	18.1	27.1	27.1	27.1	27.1	27.1	27.1	16.7	16.4	22.1	17	17.4	16.3	16.8	18.3	17.9	25.3	25.3	50	50	50	50.6	100	46.4	46.4	15.5	2.6	2.6	2.6	1.3	1.3	1.3	2.6	0	1.3	5.5
LINCOLNB	21.6	22.3	22.6	22.4	23.4	23.4	22.4	22.4	21.1	28.8	21.3	21.7	21.7	22.1	22.7	19.3	19.3	19.3	19.3	19.3	19.3	22.4	22.1	18.8	18.2	20.3	20.4	19.3	18.1	20.3	28.8	28.8	41.5	41.5	41.5	41.9	46.4	100	100	13.9	2.6	2.6	2.6	1.3	1.3	1.3	1.4	2.5	13.6
WANDERER	21.6	22.3	22.6	22.4	23.4	23.4	22.4	22.4	21.1	28.8	21.3	21.7	21.7	22.1	22.7	19.3	19.3	19.3	19.3	19.3	19.3	22.4	22.1	18.8	18.2	20.3	20.4	19.3	18.1	20.3	28.8	28.8	41.5	41.5	41.5	41.9	46.4	100	100	13.9	2.6	2.6	2.6	1.3	1.3	1.3	1.4	2.5	13.6
LILY	28	28.9	29.4	30.6	29.9	29.9	29.1	29.1	28.8	29.4	29.6	30.2	30.2	28.7	28.5	39	39	39	39	39	39	36.4	31.5	31	28.6	32	37	34.9	32.4	34.2	17.8	17.8	12.6	12.6	12.6	12.7	15.5	13.9	13.9	100	1.3	1.3	1.3	1.2	1.2	2.5	0	3.7	5.3
ASH	1.3	1.4	1.4	2.7	5.3	5.3	4.1	4.1	1.3	2.6	4.2	4.2	4.2	5.4	4.5	2.7	2.7	2.7	2.7	2.7	2.7	4.1	4	5.3	5.1	1.4	2.7	2.5	5	4.9	1.2	1.2	2.3	2.3	2.3	2.4	2.6	2.6	2.6	1.3	100	100	99.4	81.6	81.6	81.2	83	76.2	4.9
LEY	1.3	1.4	1.4	2.7	5.3	5.3	4.1	4.1	1.3	2.6	4.2	4.2	4.2	5.4	4.5	2.7	2.7	2.7	2.7	2.7	2.7	4.1	4	5.3	5.1	1.4	2.7	2.5	5	4.9	1.2	1.2	2.3	2.3	2.3	2.4	2.6	2.6	2.6	1.3	100	100	99.4	81.6	81.6	81.2	83	76.2	4.9
C7CDELTA	1.3	1.4	1.4	2.7	5.3	5.3	4.1	4.1	1.3	2.6	4.2	4.3	4.3	5.4	4.5	2.7	2.7	2.7	2.7	2.7	2.7	4.1	4	5.3	5.1	1.4	2.7	2.5	5.1	4.9	1.2	1.2	2.3	2.3	2.3	2.4	2.6	2.6	2.6	1.3	99.4	99.4	100	80.9	80.9	80.5	82.2	76.6	5
HALCYONE	2.6	2.7	2.7	4	3.9	3.9	2.7	2.7	5.1	2.6	2.7	2.8	2.8	2.7	3	2.7	2.7	2.7	2.7	2.7	2.7	4	2.7	3.9	3.8	1.4	2.6	2.5	3.7	2.4	1.2	1.2	3.4	3.4	3.4	3.5	1.3	1.3	1.3	1.2	81.6	81.6	80.9	100	100	92.7	89.5	70.8	3.7
HEATH	2.6	2.7	2.7	4	3.9	3.9	2.7	2.7	5.1	2.6	2.7	2.8	2.8	2.7	3	2.7	2.7	2.7	2.7	2.7	2.7	4	2.7	3.9	3.8	1.4	2.6	2.5	3.7	2.4	1.2	1.2	3.4	3.4	3.4	3.5	1.3	1.3	1.3	1.2	81.6	81.6	80.9	100	100	92.7	89.5	70.8	3.7
SCOTTIE	2.6	2.7	2.7	4	3.9	3.9	2.7	2.7	5	2.6	2.7	2.8	2.8	2.6	2.9	2.7	2.7	2.7	2.7	2.7	2.7	4	2.6	3.9	3.7	1.4	2.6	2.5	3.7	2.4	1.2	1.2	2.3	2.3	2.3	2.3	2.6	1.3	1.3	2.5	81.2	81.2	80.5	92.7	92.7	100	84.2	70.4	3.6
UNITY	1.4	1.4	1.5	2.9	2.8	2.8	1.4	1.4	1.4	1.4	1.5	1.5	1.5	1.4	1.6	1.4	1.4	1.4	1.4	1.4	1.4	2.9	2.8	2.8	1.3	1.5	1.4	1.3	1.3	1.3	1.3	1.3	0	0	0	0	0	1.4	1.4	0	83	83	82.2	89.5	89.5	84.2	100	70.4	3.9
TRIPP	2.6	2.6	2.7	4	3.9	3.9	2.7	2.7	2.5	2.6	4.1	4.2	4.2	3.9	4.4	1.3	1.3	1.3	1.3	1.3	1.3	7	5.3	3.9	5	2.8	5.2	4.9	6.2	4.8	4.6	4.6	2.3	2.3	2.3	2.3	1.3	2.5	2.5	3.7	76.2	76.2	76.6	70.8	70.8	70.4	70.4	100	3.6
API480	4.1	4.3	4.3	4.3	5.6	5.6	5.7	5.7	4	8.2	4.4	4.4	4.4	4.2	4.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7	7	4.2	4	4.5	5.6	5.3	5.3	5.2	11.2	11.2	9.8	9.8	9.8	10	5.5	13.6	13.6	5.3	4.9	4.9	5	3.7	3.7	3.6	3.9	3.6	100

Table 8: Function and distribution of all API480 genes (n=19) shared with *P. larvae* phages, according to Phamerator. For each API480 gene product (pham) that is shared, the number of phages per cluster that have protein homologs are listed. The total number of phages that belong to each cluster are in brackets. Below, the percentage of phages for each cluster that shared proteins with API480 is provided.

API480 Genome		Clusters				
Protein	**Function**	**Fern (n=30)**	**Harrison (n=2)**	**Vegas (n=7)**	**Lily (n=1)**	**Halcyone (n=8)**
gp17	Holin	30	2	7	1	0
gp18	Endolysin	30	2	7	1	0
gp19	Hypotethical protein	0	0	4	0	0
gp20	Hypotethical protein	1	2	2	0	0
gp21	Holin	30	2	7	1	0
gp30	Hypothetical protein	0	0	4	0	0
gp34	Hypothetical protein	0	0	0	0	8
gp38	Hypotethical protein	6	0	0	3	3
gp37	Hypotethical protein	0	0	2	0	7
gp39	dUTP diphosphatase	0	0	0	0	7
gp40	Hypotethical protein	0	0	4	0	0
gp41	Hypotethical protein	0	0	4	0	0
gp42	Hypotethical protein	0	0	0	0	1
gp48	Dingle stranded binding protein	0	2	0	0	0
gp50	Host-nuclease inhibitor	0	2	0	0	0
gp52	Hypotethical protein	1	0	2	0	0
gp53	Hypothetical protein	0	2	2	0	1
gp55	Hypothetical protein	11	2	2	1	0
gp73	Hypothetical protein	1	2	6	0	0
		19 %	**47 %**	**39 %**	**37 %**	**18 %**

3.3.3.3 Mass spectrometry

To confirm API480 gene predictions, its proteome was analysed by mass spectrometry. An SDS-PAGE gel loaded with unpurified phage particles showed several bands (**Figure 18**). Thirteen gel fragments were extracted and further sequenced. A total of 47 proteins could be identified (**Table 9**). From the 28 unique proteins from API480, 11 revealed an amino acidic sequence coverage ranging from 24.3% to 69.5%. A total of 29 proteins presented homology to bacterial proteins, from which 20 had a cover range between 7.9% and 93.7%. Finally, from the 20 proteins with homology to other phage, 16 displayed a cover range between 12.5% and 89.2%.

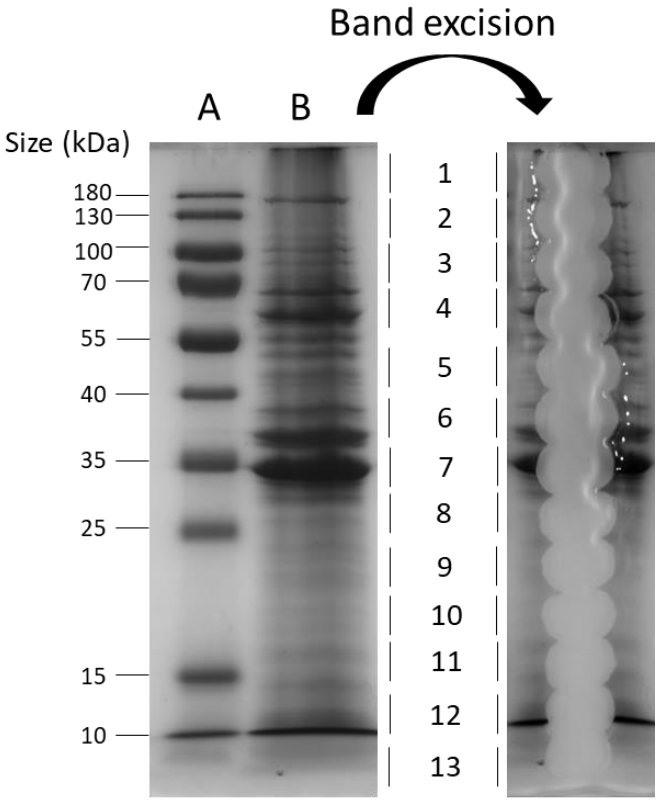

Figure 18: SDS-PAGE analysis of the API480 phage proteins (lane B) on a 12% SDS-PAGE separation gel. Lane A shows the protein ladder bands from 10 to180 kDa. The entire lane B was cut into 13 slices, numbered on the right. Band excision is illustrated in the cropped image from the same gel, as indicated.

Table 9: Bacteriophage API480 proteins identified by ESI-MS/MS, after denaturation and phage particle fractionation on SDS-PAGE gel. Identified phage proteins are listed below. SDS-PAGE gel band in which the proteins were identified have been indicated as well as the protein mass, the number of identified unique peptides and the protein sequence that is covered by the peptide (in %).

Protein	Identified Function	Band No. (most abundant)	Protein MW (kDa)	No. of unique peptides	Sequence coverage (%)
gp1		11,12 (11)	16.55	6	50.40
gp2		11,12 (12)	9.96	4	58.30
gp3		5,12 (12)	11.95	6	55.90
gp4	Terminase large subunit	1,2,3,4,5,6,8,9,10,11 (1)	55.47	14	36.66
gp5		1,2,5,10,11,12 (12)	7.05	3	38.10
gp6	Portal protein	1,2,3,4,5,6,7,8,9,10,11,12 (3)	68.14	35	62.68
gp7		1,2,3,4,5,6,7,8,9,10,11,12 (6)	31.45	31	93.70
gp8	Major capsid protein	1,2,3,4,5,6,7,8,9,10,11,12 (7)	34.38	34	89.16
gp9		5,9,12,13 (12)	9.88	7	67.80
gp10		11,12,13 (12)	14.40	5	67.50
gp11		1,2,3,4,12(3)	75.23	12	24.26
gp12		1,2,3,4,5,6,8,9,11 (5)	43.56	17	50.75
gp13		12	11.64	7	68.00
gp14		2,6,7,8,9,10,11 (6)	35.47	18	55.71
gp15		6,7,8,9,10,11,12 (6)	37.05	19	63.00
gp16		1,2,3,4,5,6,7,8,9,10,11,12 (2)	128.32	71	60.75
gp17	Holin	12	9.95	4	35.00
gp20		12	12.52	3	42.90
gp21	Holin	12	8.27	1	26.60
gp24		1,2,6,9,12 (1)	36.60	5	15.58
gp26	mRNA-degrading endonuclease	12	12.52	3	28.40
gp30		11	14.13	1	13.70
gp34		12	9.76	1	16.30
gp36		10	14.43	1	7.94
gp37		12	10.62	3	59.40
gp38		12	12.33	2	37.40
gp39	Deoxyuridine 5'-triphosphate nucleotidohydrolase	1,2,3,4,5,6,8,9,10,12 (10)	18.21	7	49.69
gp40		10,11,12 (12)	9.82	6	66.30
gp43		12	7.15	5	87.90
gp44	Resolvase	1,2,3,4,5,6,7,8,9,10,11,12 (11)	19.12	8	56.90
gp46	DNA polymerase I	1,2,3,4 (3)	87.14	28	42.96
gp47	DNA primase	1,2,3 (1)	105.39	9	12.50
gp48	Dingle-stranded DNA-binding protein	3,5,8,9,10,11,12 (10)	17.60	11	62.10

gp49		1,8,9,10,11,12 (9)	20.18	13	69.48
gp50	Host-nuclease inhibitor	8,9,10 (8)	21,75	10	60.99
gp53		11,12 (12)	8.58	1	18.40
gp54		12	11.83	3	41.20
gp55		12	6.91	2	53.30
gp57	DEAD/DEAH box helicase	1,2,4,8 (1)	60.63	7	20.10
gp59		8	25,95	7	44.70
gp60		11,12 (11)	19.76	3	18.00
gp63		12	9.65	2	31.30
gp64		12	7.95	2	37.30
gp65		1,11,12 (11)	15.46	6	65.39
gp70	Antirestriction protein ArdA	9	18,92	3	19.90
gp76		1,11,12 (11)	17.36	7	59.60
gp77		12,13 (12)	8.14	4	63.40

3.3.4 Phage potential for biocontrol application in hives

The potential of the therapeutic use of API480 in apiaries, as far as AFB control is concerned, was investigated through the assessment of the phage growth dynamic, life cycle and stability on field conditions.

3.3.4.1 Phage integration assays

Although no integrase was identified in API480 genome by the *in silico* analysis, the ability of the phage to lysogenise its host was investigated to obtain more consistent information about the phage cycle. Assuming that a lysogenized host might become resistant to the recently integrated phage by acquiring phage genetic material, the infectivity of API480 was assessed after lysogeny induction together with the presence of phage genes in host resistant colonies.

Contrary to the original PI02-27 strain, results revealed that the phage lost the ability to infect R-PI27 strains, and that the DNA of the same strains allowed the amplification of a 227 bp band (specific for API480) by PCR (**Figure 19**).

To assess if the use of a phage cocktail could be relevant in supporting API480 in AFB infections, the activity of the other five *P. larvae* phages from our collection against R-PI27 colonies was investigated and all of them were infective.

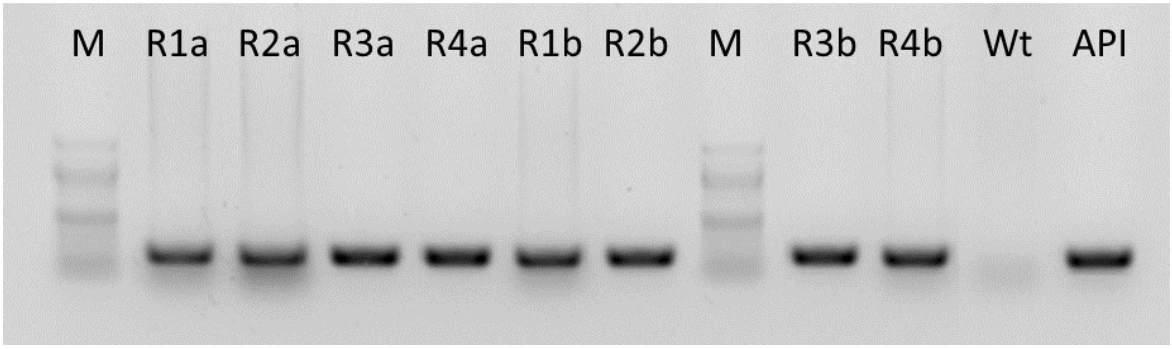

Figure 19: Amplification of CDS_12 fragment. Agarose gel revealing 227 bp bands in R-PI27 strains tested (R1, R2, R3 and R4 in duplicate (a and b), in API480 (API) and it absence in the original PI02-27 strain (Wt: wild type). DNA ladder 100 bp NEB (M).

3.3.4.2 Phage infection parameters

The assessment of the phage generation time and the phage population growth level was accomplished through the determination of phage infection parameters.

Adsorption assays revealed that during the first minutes of phage contact with its host, the number of free phages rapidly decreased. After 35 minutes approximately 85% of the total API480 phage particles were adsorbed to its host (**Figure 20A**).

As far as phage growth cycle parameters are concerned, the calculated latent period was approximately 30 minutes and the phage burst size was approximately 3 PFU per infected cell (**Figure 20B**).

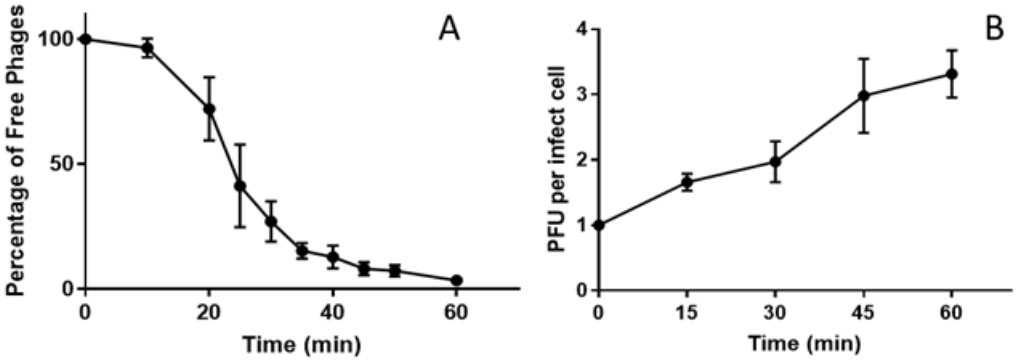

Figure 20: Phage-host interaction parameters. (A) Percentage of free API480 phages after infection of *P. larvae* (MOI=0.1). (B) One-step growth curve of phage API480 in *P. larvae* PI02–27. Shown are the PFU per infected cell. Each point represents the average of three independent assays and error bars indicate the standard deviation. Statistical significance, $p<0.05$.

3.3.4.3 Phage stability in simulated filed conditions

The API480 phage stability in a 50% (w/v) sucrose solution was assessed *in vitro* for 24 hours. The results revealed that, at least in this time period, the phage viability was not impacted (**Figure 21**).

The evaluation of phage viability in RJ (pH 4.0) revealed a total loss of phage infectivity after 6 hours (**Figure 22A**).

The impact of low pH solutions (3.0, 3.5, 4.0, 4.5 and 5.0) in API480 stability was also assessed. Results, presented in **Figure 22B**, revealed that after 6 hours the phage was completely inactivated at pH 3.0 (5.5 Log reduction in average). Interestingly, at pH 3.5, at the same time point, it only caused a slight decrease of 0.1 Log PFU.mL^{-1}, being accentuated ($p<0.05$) after 24 hours, with an average reduction of 4.3 Log PFU.mL^{-1}. No effect was observed with the other pH values when assessed for periods lower than 24 hours. Nevertheless, at this time point, the average phage lost was of 2.7, 0.8 and 0.6 Log PFU.mL^{-1} at pH 4.0, 4.5 and 5.0, respectively.

The assessment of the phage stability in larvae homogenised revealed that a slight and not statistically meaningful phage reduction of 0.4 Log PFU.mL^{-1} ($p>0.05$) was observed, and only after 24 hours (**Figure 23**). However, in the crop and mid- hindgut (homogenized tissues) the phage suffered losses in its viability. The phage reduction in crop were of 1.2 and 3.2 Log PFU.mL^{-1} in 6 and 24 hours respectively. Regarding to mid- hindgut the phage reductions were 0.1 and 1.6 Log PFU.mL^{-1} in 6 and 24 hours respectively (**Figure 23**). Mid- hindguts are less aggressive than the crops to API480 phage.

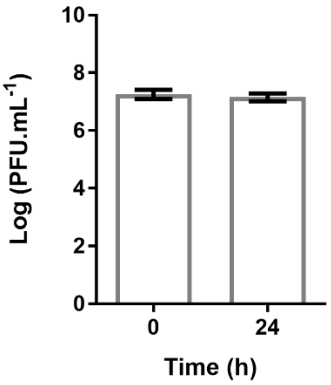

Figure 21: Effect of 50% (w/v) sucrose on API480 phage concentration (PFU.mL^{-1}). Data shows represents the average of three independent assays and error bars indicate the standard deviation. LOD (Limit of Detection) = 3 Log. Statistical significance, $p<0.05$.

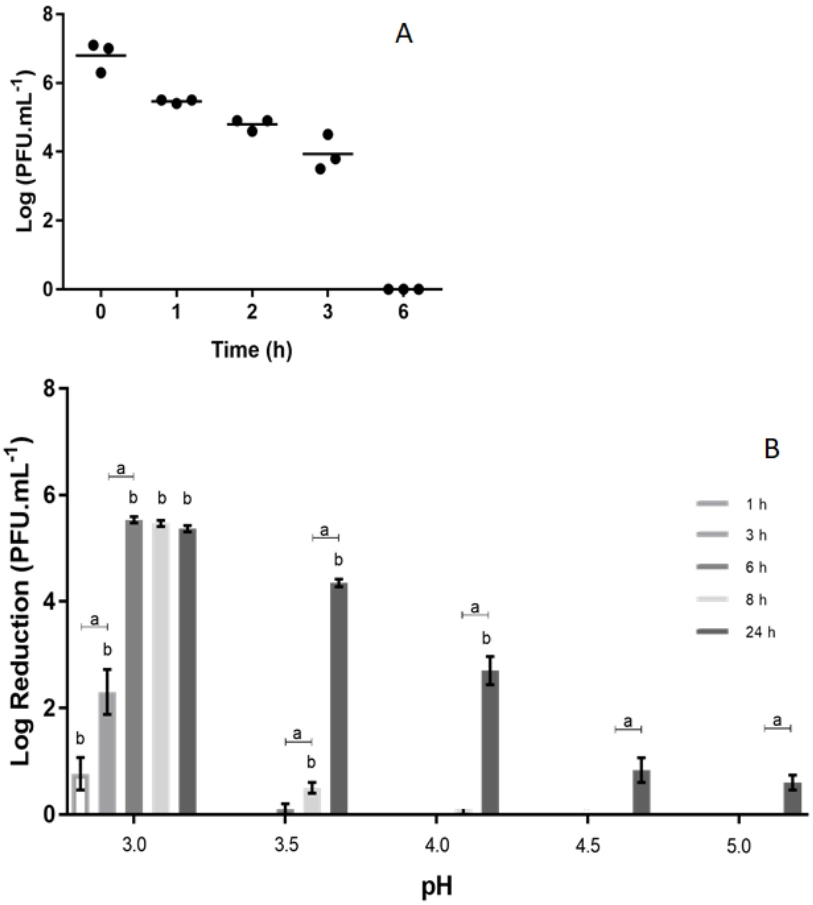

Figure 22: (A) Effect of commercial RJ on the stability of API480 (PFU.mL^{-1}). Data show each of three independent assays. Limit of detection (LOD) = 3 Log; Statistical significance, $p<0.05$. (B) Effect of pH (from 3.0 to 5.0) in API480 phage concentration (PFU.mL^{-1}). Each column represents the average of three independent assays and error bars indicate the standard deviation. LOD = 1 Log; Statistical significance, $p<0.05$; "a" indicates differences for the same pH; for each timepoint, "b" indicates differences between data from a given pH and the subsequent pH value.

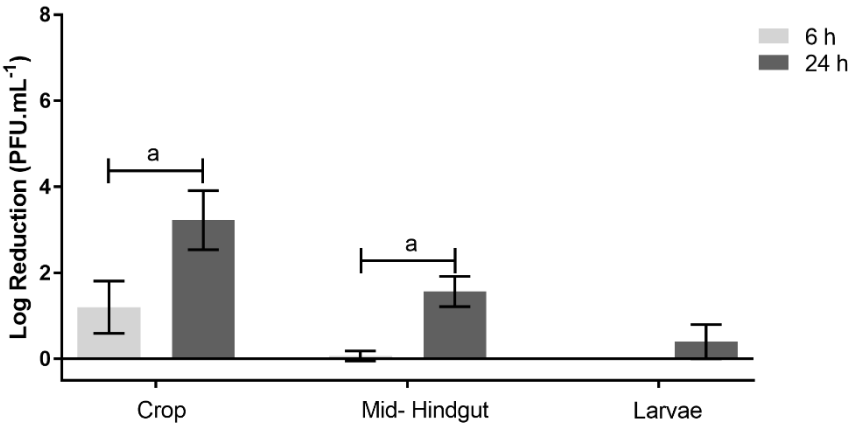

Figure 23: Effect of homogenized crop, mid- hindgut and larvae tissue on the stability of API480 (PFU.mL^{-1}) after 6 and 24 hours in contact. Each column represents the average of three independent assays and error bars indicate the standard deviation. LOD = 1 Log; Statistical significance, $p<0.05$; "a" indicates differences between the two timepoints.

3.4 Discussion

The isolation of the first genomic sequence of a *P. larvae* podovirus, vB_PlaP_API480 (API480) is described here. So far, all *P. larvae* phages (n=48) have been reported to be siphoviruses, with a temperate lifestyle and the majority (n=40) with a wide genetic similarity. The only exception is the Halcyone cluster (n=8) that is quite diverse from other *P. larvae* phages (Stamereilers et al., 2018). We showed that API480 has very limited genomic and proteomic relatedness to any phage deposited in public databases, including *P. larvae* phages. From the 77 CDS predicted in the phage genome, 47 were confirmed by MS/MS. Genomic comparison analysis demonstrated that API480 is a singleton with 28 unique proteins, sharing only 19 of its proteins with other *P. larvae* siphoviruses, mostly with Harrison and Vegas clusters. This can be interpreted as a recent evolutionary history between API480 and these phages. It was surprising to see how the API480 host lysis proteins seem to be composed of one endolysin and two putative holins and how these proteins are the most conserved among *P. larvae* phages. Although these putative holins need to be experimentally confirmed, they apparently provide a new way for phages to lyse its host cells at the end of its lytic cycle.

As the majority of phages known today, API480 and the other *P. larvae* phages also have their genomes organised in mosaics, starting with the structural and morphogenetic modules that are followed by the host lysis, DNA replication and metabolism related proteins. They all seem to have an overall common structure despite the limited shared gene content between them. This reinforces the existence of a common evolutionary ancestor among these phages. The low frequency of singletons found in *P. larvae* phages (from n=49, 4% are singletons) which differs from other phage populations infecting hosts of similar taxonomic level, such as *Bacillus* (from n=83, 18.1% are singletons) (Grose, Jensen, Burnett, & Breakwell, 2014) and *Gordonia* phages (from n=79, 17.7% are singletons) (Pope et al., 2017), indicates that the full genetic diversity is still untapped. This can be the result of similar isolation techniques used but not the outcome of low environmental sampling diversity, as *P. larvae* phages have been isolated from different isolation sources and geographical regions (Stamereilers et al., 2018). Overall, the genomic and proteomic analysis show that API480 is a completely distinct phage, suggesting the creation of a new genus within the *Podoviridae* family.

The analysis of the firstly reported genetic sequence of API480, widely distinct from the others (maximum genome coverage of 5%), encouraged its detailed characterization considering its use in AFB control.

The evaluation of API480 life cycle was accomplished based on the *in silico* analysis together with the assessment of the phage ability to integrate the bacterial host genome. The ability to lysogenise

bacteria by a temperate phage is based on a gene cluster responsible for integration (integrase) and maintenance (repressors of the lytic cycle) of the prophage. Unlike all the other *P. larvae* phages no lysogeny module and no integrase gene were found in API480 genome.

The temperate nature of the phage was confirmed by the detection of a phage gene in the host genome and by the conversion of PI02-27 into a phage-resistant strain. Although the use of temperate phages for therapeutic purposes is accepted, it raises several concerns (J. Y. Nale, Spencer, et al., 2016). Some strategies are known to safeguard its use for this purpose based on the difficulty of isolating strictly lytic phages, which was already reported for other bacterial species. Recently, Nale et al. (2018) revealed that all published *Clostridium difficile* phages are so far temperate and encode an integrase gene (J. Nale et al., 2018). Meader and colleagues (2010) anticipated that this is possibly due to the high incidence of prophage genes, revealing resistance to further infections (Meader et al., 2010). They also infer that the spore form may favour phage integration into the genome.

The use of optimized phage combinations with distinct and often complementary features (such as host range), as a strategy to lighten the effect of lysogeny and consequent phage resistance, has already been demonstrated (J. Y. Nale, Spencer, et al., 2016). The susceptibility of R-PI27 strains to other *P. larvae* phages from our collection confirmed this idea.

Recent reports on the use of temperate phages for therapy were recently reviewed (Monteiro et al., 2018). For example, phage ØCD27 was used to control *C. difficile* infections reducing their presence in the colon and decreasing the toxin expression (Meader et al., 2013). Nale et al. (2016) reported the reduction of *C. difficile* colonisation by a cocktail of temperate phages extending the life expectancy of mice (J. Y. Nale, Spencer, et al., 2016). A *Pseudomonas aeruginosa* systemic infection was treated in flies and in a murine animal model using two temperate phages (MP22 and D3112) (Chung, Sim, & Cho, 2012).

As far as AFB control is concerned, spore infected lab-reared larvae were also successfully treated with cocktails of *P. larvae* phages with a temperate lifestyle (Ghorbani-Nezami et al., 2015; Yost et al., 2016).

API480 revealed to have broad lytic spectra, being active against 69% of the isolated field strains. All these strains belong to ERIC I, the far more diverse worldwide genotype (Morrissey et al., 2015) and the one that causes higher morbidity in hives (Rauch et al., 2009). API480 also seems to be able to infect and propagate in ERIC II strains. Nevertheless, no evidence was found that the phage can be used to control infections caused by ERIC III and IV strains, as in this case the lysis occur without phage replication.

According to Yost et al. (2016) phages show host preference for the ERIC group from which they were isolated, and the preference for ERIC I was also observed in other *P. larvae* phages (Yost et al., 2016).

The high specificity that this phage revealed for *P. larvae* is particularly important as far as the first-instar larval commensal bacteria*, L. kunkeei* and *P. apium* alpha 2.2 are concerned, as it indicates that this phage would not have a harmful impact on their gut microbiota (Corby-Harris et al., 2014; Vojvodic et al., 2013).

While studying the phage growth parameters, API480 revealed a slow adsorption to its host (in 35 minutes) as reported for example in the *C. difficile* phage CDHM1 (30 minutes) (Thanki et al., 2018) and a release of as few as three progeny viruses per infected cell. A burst of around eight phages per cell was recently reported in the *P. larvae* phage HB10c2 (Beims et al., 2015) and a burst of five and seven phages per cell was obtained in other two temperate phages isolated from *C. difficile*, φC2 and φC5, respectively (Goh, Riley, & Chang, 2005). The latter authors hypothesised that these result could be due to suboptimal growth conditions, explanation also given by Touchon et al. (2016) while studying life-history traits of temperate phages associated with lysogeny (Touchon, Bernheim, & Rocha, 2016). According to a study carried out by Hadas et al. (1997) with T4 phage, parameters of phage development and cell lysis are dependent on bacterial growth rate (Hadas, Einav, & Zaritsky, 1997). Correspondingly, slow adsorption rates, small burst size and high latent period were expected in a slow-grower bacterium such as *P. larvae* (Garcia-Gonzalez, Poppinga, et al., 2014).

The stability of API480 in simulated hive conditions was assessed to learn about its suitability in AFB biocontrol. API480 was very stable when exposed to high glucose concentrations (frequently used for feeding bees) and to pH values higher than 4.0 (often found in the hive).

Moreover, the phage infectivity was almost unaffected by larval fluids (0.4 Log $PFU.mL^{-1}$ reduction) indicating that, at least for 24 hours, this is a favourable compartment for the host infection. Nevertheless, before reaching the larvae, phages will be mixed with RJ during the crop content regurgitation back to the mouth (Hrncir & Jarau, 2009) and they will be available in brood combs for larval consumption. According to our results, larvae have no more than 6 hours to ingest phages in a viable state. The decrease in phage viability at some point was an expected result, as Ribeiro et al., (2019) have recently reported. Some RJ elements may be responsible for this antiviral effect, such as proteinases (Chen, 1995) and phenolic compounds (Pasupuleti et al., 2017) that probably interact with phage structural proteins, contributing to their inactivation (Ali et al., 2012). The crop section is also characterized as acidic environment that affect

the phage viability, which indicates phage needs some environment protection as previous mentioned by Ribeiro et al. (2019) (Ribeiro, Correia, et al., 2019).

In conclusion, despite being a temperate phage, the API480 broad lytic spectra, the specificity to *P. larvae* and the behaviour when challenged by simulated hive conditions encouraged its use for therapeutic purposes. Furthermore, the possibility of administering it together with other *P. larvae* phages which are able to infect API480 resistant strains mitigates the lysogenic nature of the phage.

References

Ali, H., Alli, I., Ismail, A., & Kermasha, S. (2012). Protein-phenolic interactions in food. *Eurasian Journal of Analytical Chemistry*, *7*(3), 123–133.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Anderson, K. E., Sheehan, T. H., Mott, B. M., Maes, P., Snyder, L., Schwan, M. R., ... Corby-Harris, V. (2013). Microbial ecology of the hive and pollination landscape: Bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (Apis mellifera). *PLoS ONE*, *8*(12), e83125. https://doi.org/10.1371/journal.pone.0083125

Atterbury, R. J. (2009). Bacteriophage biocontrol in animals and meat products, *2*, 601–612. https://doi.org/10.1111/j.1751-7915.2009.00089.x

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., ... Zagnitko, O. (2008). The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics*, *9*(1), 75. https://doi.org/10.1186/1471-2164-9-75

Beims, H., Wittmann, J., Bunk, B., Spröer, C., Rohde, C., Günther, G., ... Steinert, M. (2015). Paenibacillus larvae-Directed bacteriophage HB10c2 and its application in American foulbrood-affected honey bee larvae. *Applied and Environmental Microbiology*, *81*(16), 5411–5419. https://doi.org/10.1128/AEM.00804-15

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of Paenibacillus larvae in honeybee hives. *Journal of Invertebrate Pathology*, *150*(September), 94–100. https://doi.org/10.1016/j.jip.2017.09.010

Chen, C. (1995). Changes in protein components and storage stability of royal jelly under various conditions. *Food Chemistry*, *54*(2), 195–200. https://doi.org/10.1016/0308-8146(95)00031-D

Chung, I., Sim, N., & Cho, Y. (2012). Antibacterial Efficacy of Temperate Phage-Mediated Inhibition of Bacterial Group Motilities. *Antimicrobial Agents and Chemotherapy*, *56*(11), 5612–5617. https://doi.org/10.1128/AAC.00504-12

Colibar, O., Popovici, D., Eugeniu, C., & Korodi, G. (2010). The effect of acidifiant on the development of bee families (Apis mellifica). *Med. Vet.*, *43*(2), 296–299.

Corby-Harris, V., Snyder, L. A., Schwan, M. R., Maes, P., McFrederick, Q. S., & Anderson, K. E. (2014). Origin and effect of Alpha 2.2 Acetobacteraceae in honey bee larvae and description of Parasaccharibacter apium gen. nov., sp. nov. *Applied and Environmental Microbiology*, *80*(24), 7460–7472. https://doi.org/10.1128/AEM.02043-14

Crailsheim, K., & Riessberger-Gallé, U. (2001). Honey bee age-dependent resistance against American foulbrood. *Apidologie*, *32*(1), 91–103. https://doi.org/10.1051/apido:2001114

Cresawn, S. G., Bogel, M., Day, N., Jacobs-Sera, D., Hendrix, R. W., & Hatfull, G. F. (2011). Phamerator: A bioinformatic tool for comparative bacteriophage genomics. *BMC Bioinformatics*, *12*(1), 395. https://doi.org/10.1186/1471-2105-12-395

Dobbelaere, W., de Graaf, D. C., & Peeters, J. E. (2001). Development of a fast and reliable diagnostic method for American foulbrood disease (Paenibacillus larvae subsp. larvae) using a 16S rRNA gene based PCR. *Apidologie*,

32(4), 363–370. https://doi.org/10.1051/apido:2001136

Drulis-Kawa, Z., Majkowska-Skrobek, G., & Maciejewska, B. (2015). Bacteriophages and phage-derived proteins – Application approaches. *Current Medicinal Chemistry*, *22*(14), 1757–1773. https://doi.org/10.2174/0929867322666150209152851

European Parliament and the Council of the European Union. (2010). Commission regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuff of animal origin. *Official Journal of the European Union*, (L 15/1).

Garcia-Gonzalez, E., Poppinga, L., Fünfhaus, A., Hertlein, G., Hedtke, K., Jakubowska, A., & Genersch, E. (2014). Paenibacillus larvae Chitin-Degrading Protein PlCBP49 Is a Key Virulence Factor in American Foulbrood of Honey Bees. *PLoS Pathogens*, *10*(7), e1004284. https://doi.org/10.1371/journal.ppat.1004284

Genersch, E. (2010). American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. *Journal of Invertebrate Pathology*, *103*(SUPPL. 1), S10–S19. https://doi.org/10.1016/j.jip.2009.06.015

Genersch, E., Ashiralieva, A., & Fries, I. (2005). Strain- and Genotype-Specific Differences in Virulence of Paenibacillus larvae subsp. larvae, a Bacterial Pathogen Causing American Foulbrood Disease in Honeybees. *Applied and Environmental Microbiology*, *71*(11), 7551–7555. https://doi.org/10.1128/AEM.71.11.7551-7555.2005

Genersch, E., & Otten, C. (2003). The use of repetitive element PCR fingerprinting (rep-PCR) for genetic subtyping of German field isolates of Paenibacillus larvae subsp. larvae. *Apidologie*, *34*(3), 195–206. https://doi.org/10.1051/apido:2003025

Ghorbani-Nezami, S., LeBlanc, L., Yost, D. G., & Amy, P. S. (2015). Phage Therapy is Effective in Protecting Honeybee Larvae from American Foulbrood Disease. *Journal of Insect Science*, *15*(1), 84. https://doi.org/10.1093/jisesa/iev051

Goh, S., Riley, T. V., & Chang, B. J. (2005). Isolation and Characterization of Temperate Bacteriophages of Clostridium difficile. *Applied and Environmental Microbiology*, *71*(2), 1079–1083. https://doi.org/10.1128/AEM.71.2.1079-1083.2005

Grose, J. H., Jensen, G. L., Burnett, S. H., & Breakwell, D. P. (2014). Genomic comparison of 93 Bacillus phages reveals 12 clusters, 14 singletons and remarkable diversity. *BMC Genomics*, *15*(1), 855. https://doi.org/10.1186/1471-2164-15-855

Hadas, H., Einav, M., & Zaritsky, A. (1997). Bacteriophage T4 development depends on the physiology of its host Escherichia coli. *Microbiology*, (May), 179–185.

Hrncir, M., & Jarau, S. (2009). *Food Exploitation by Social Insects - Ecological, Behavioral, and Theoretical Approaches*. *Food Exploitation By Social Insects*. https://doi.org/doi:10.1201/9781420075618.bmatt

Huson, D. H. (1998). SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics*, *14*(1), 68–73. https://doi.org/10.1093/bioinformatics/14.1.68

Kalatzis, P. G., Rørbo, N., Castillo, D., Mauritzen, J. J., Jørgensen, J., Kokkari, C., … Middelboe, M. (2017). Stumbling across the same phage: Comparative genomics of widespread temperate phages infecting the fish pathogen Vibrio anguillarum. *Viruses*, *9*(5), 1–19. https://doi.org/10.3390/v9050122

Käll, L., & Sonnhammer, E. L. . (2002). Reliability of transmembrane predictions in whole-genome data. *FEBS Letters*, *532*(3), 415–418. https://doi.org/10.1016/S0014-5793(02)03730-4

Klucar, L., Stano, M., & Hajduk, M. (2010). phiSITE: database of gene regulation in bacteriophages. *Nucleic Acids*

Research, *38*(Database), D366–D370. https://doi.org/10.1093/nar/gkp911

Meader, E., Mayer, M. J., Gasson, M. J., Steverding, D., Carding, S. R., & Narbad, A. (2010). Bacteriophage treatment significantly reduces viable Clostridium difficile and prevents toxin production in an in vitro model system. *Anaerobe*, *16*(6), 549–554. https://doi.org/10.1016/j.anaerobe.2010.08.006

Meader, E., Mayer, M. J., Steverding, D., Carding, S. R., & Narbad, A. (2013). Evaluation of bacteriophage therapy to control Clostridium difficile and toxin production in an in vitro human colon model system. *Anaerobe*, *22*, 25–30. https://doi.org/10.1016/j.anaerobe.2013.05.001

Melo, L. D. R., Sillankorva, S., Ackermann, H.-W., Kropinski, A. M., Azeredo, J., & Cerca, N. (2014). Isolation and characterization of a new Staphylococcus epidermidis broad-spectrum bacteriophage. *Journal of General Virology*, *95*(Pt_2), 506–515. https://doi.org/10.1099/vir.0.060590-0

Miyagi, T., Peng, C. Y. S., Chuang, R. Y., Mussen, E. C., Spivak, M. S., & Doi, R. H. (2000). Verification of oxytetracycline-resistant American foulbrood pathogen Paenibacillus larvae in the United States. *Journal of Invertebrate Pathology*, *75*(1), 95–96. https://doi.org/10.1006/jipa.1999.4888

Monteiro, R., Pires, D. P., Costa, A. R., & Azeredo, J. (2018). Phage Therapy: Going Temperate? *Trends in Microbiology*, *xx*, 1–11. https://doi.org/10.1016/j.tim.2018.10.008

Morrissey, B. J., Helgason, T., Poppinga, L., F??nfhaus, A., Genersch, E., & Budge, G. E. (2015). Biogeography of Paenibacillus larvae, the causative agent of American foulbrood, using a new multilocus sequence typing scheme. *Environmental Microbiology*, *17*(4), 1414–1424. https://doi.org/10.1111/1462-2920.12625

Nale, J., Redgwell, T., Millard, A., & Clokie, M. (2018). Efficacy of an Optimised Bacteriophage Cocktail to Clear Clostridium difficile in a Batch Fermentation Model. *Antibiotics*, *7*(1), 13. https://doi.org/10.3390/antibiotics7010013

Nale, J. Y., Spencer, J., Hargreaves, K. R., Buckley, A. M., Trzepin, P., & Douce, G. R. (2016). Bacteriophage Combinations Significantly Reduce Clostridium difficile Growth In Vitro and Proliferation In Vivo. *Antimicrobial Agents and Chemotherapy*, *60*(2), 968–981. https://doi.org/10.1128/AAC.01774-15.Address

Naville, M., Ghuillot-Gaudeffroy, A., Marchais, A., & Gautheret, D. (2011). ARNold: A web tool for the prediction of Rho-independent transcription terminators. *RNA Biology*, *8*(1), 11–13. https://doi.org/10.4161/rna.8.1.13346

Oliveira, A., Leite, M., Kluskens, L. D., Santos, S. B., Melo, L. D. R., & Azeredo, J. (2015). The First Paenibacillus larvae Bacteriophage Endolysin (PlyPl23) with High Potential to Control American Foulbrood. *PLOS ONE*, *10*(7), e0132095. https://doi.org/10.1371/journal.pone.0132095

Oliveira, A., Melo, L. D. R., Kropinski, A. M., & Azeredo, J. (2013). Complete Genome Sequence of the Broad-Host-Range Paenibacillus larvae Phage phiIBB_Pl23. *Genome Announcements*, *1*(5), e00438-13-e00438-13. https://doi.org/10.1128/genomeA.00438-13

Oliveira, H., Costa, A. R., Ferreira, A., Konstantinides, N., Santos, S. B., Boon, M., ... Azeredo, J. (2018). Functional Analysis and Antivirulence Properties of a New Depolymerase from a Myovirus That Infects Acinetobacter baumannii Capsule K45. *Journal of Virology*, *93*(4), 1–16. https://doi.org/10.1128/JVI.01163-18

Oliveira, H., Sampaio, M., Melo, L. D. R., Dias, O., Pope, W. H., Hatfull, G. F., & Azeredo, J. (2019). Staphylococci phages display vast genomic diversity and evolutionary relationships. *BMC Genomics*, *20*(1), 357. https://doi.org/10.1186/s12864-019-5647-8

Pasupuleti, V. R., Sammugam, L., Ramesh, N., & Gan, S. H. (2017). Honey, propolis, and royal jelly: A comprehensive

review of their biological actions and health benefits. *Oxidative Medicine and Cellular Longevity*, *2017*, 1–21. https://doi.org/10.1155/2017/1259510

Petersen, T. N., Brunak, S., von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods*, *8*(10), 785–786. https://doi.org/10.1038/nmeth.1701

Pires, D., Sillankorva, S., Faustino, A., & Azeredo, J. (2011). Use of newly isolated phages for control of Pseudomonas aeruginosa PAO1 and ATCC 10145 biofilms. *Research in Microbiology*, *162*(8), 798–806. https://doi.org/10.1016/j.resmic.2011.06.010

Pope, W. H., Mavrich, T. N., Garlena, R. A., Guerrero-Bustamante, C. A., Jacobs-Sera, D., Montgomery, M. T., … Hatfull, G. F. (2017). Bacteriophages of Gordonia spp. Display a Spectrum of Diversity and Genetic Relationships. *MBio*, *8*(4), 1–20. https://doi.org/10.1128/mBio.01069-17

Rauch, S., Ashiralieva, A., Hedtke, K., & Genersch, E. (2009). Negative Correlation between Individual-Insect-Level Virulence and Colony-Level Virulence of Paenibacillus larvae, the Etiological Agent of American Foulbrood of Honeybees. *Applied and Environmental Microbiology*, *75*(10), 3344–3347. https://doi.org/10.1128/AEM.02839-08

Ribeiro, H. G., Correia, R., Moreira, T., Vilas Boas, D., Azeredo, J., & Oliveira, A. (2019). Bacteriophage biodistribution and infectivity from honeybee to bee larvae using a T7 phage model. *Scientific Reports*, *9*(1), 620. https://doi.org/10.1038/s41598-018-36432-x

Schattner, P., Brooks, A. N., & Lowe, T. M. (2005). The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs. *Nucleic Acids Research*, *33*(Web Server), W686–W689. https://doi.org/10.1093/nar/gki366

Sillankorva, S., Neubauer, P., & Azeredo, J. (2008). Isolation and characterization of a T7-like lytic phage for Pseudomonas fluorescens. *BMC Biotechnology*, *8*, 80. https://doi.org/10.1186/1472-6750-8-80

Soding, J., Biegert, A., & Lupas, A. N. (2005). The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Research*, *33*(Web Server), W244–W248. https://doi.org/10.1093/nar/gki408

Stamereilers, C., Fajardo, C., Walker, J., Mendez, K., Castro-Nallar, E., Grose, J., … Tsourkas, P. (2018). Genomic Analysis of 48 Paenibacillus larvae Bacteriophages. *Viruses*, *10*(7), 377. https://doi.org/10.3390/v10070377

Thanki, A. M., Taylor-Joyce, G., Dowah, A., Nale, J. Y., Malik, D., & Clokie, M. R. J. (2018). Unravelling the Links between Phage Adsorption and Successful Infection in Clostridium difficile. *Viruses*, *10*(8), 411. https://doi.org/10.3390/v10080411

Touchon, M., Bernheim, A., & Rocha, E. P. C. (2016). Genetic and life-history traits associated with the distribution of prophages in bacteria. *The ISME Journal*, *10*(11), 2744–2754. https://doi.org/10.1038/ismej.2016.47

Vojvodic, S., Rehan, S. M., & Anderson, K. E. (2013). Microbial Gut Diversity of Africanized and European Honey Bee Larval Instars. *PLoS ONE*, *8*(8), 1–11. https://doi.org/10.1371/journal.pone.0072106

Woodrow, A. W. (1942). Susceptibility of honeybee larvae to individual inoculations with spores of *Bacillus* larvae. *Journal of Economic Entomology*, *35*(6), 892–895. https://doi.org/10.1093/jee/35.6.892

Xie, Y., Wei, Y., Shen, Y., Li, X., Zhou, H., Tai, C., … Ou, H. Y. (2018). TADB 2.0: An updated database of bacterial type II toxin-antitoxin loci. *Nucleic Acids Research*, *46*(D1), D749–D753. https://doi.org/10.1093/nar/gkx1033

Yost, D. G., Tsourkas, P., & Amy, P. S. (2016). Experimental bacteriophage treatment of honeybees (Apis mellifera)

infected with Paenibacillus larvae, the causative agent of American foulbrood disease. *Bacteriophage*, *6*(1), e1122698. https://doi.org/10.1080/21597081.2015.1122698

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., ... Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *Journal of Antimicrobial Chemotherapy*, *67*(11), 2640–2644. https://doi.org/10.1093/jac/dks261

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research*, *31*(13), 3406–3415. https://doi.org/10.1093/nar/gkg595

Supplementary information

Here, also was re-evaluated the lytic spectra of API480. In the new set of 43 field isolates all ERIC I, available after September of 2019 (Chapter 4), API480 is able to infect 34 strains (which represents 79% of the strains), however considering the all strains of the panel (n=110), the infectivity is 73%. From the 43 new strains, API480 was not able infect one strain, the H127 catalogued with EOP score of 0. The remaining eight strains identified as non-sensitive to API480 correspond to the strains where occurs LFW. Hosts with the highest EOP score of 3 (EOP > 100%) were 18, while the EOP scores of 2 and 1 had eight bacteria each **Table S10**.

Table S10: API480 lytic spectra and EOP against different strains (43 new *P. larvae* strains were obtained from dead larvae (02)). The EOP was scored as 0 (negative), 1 (<10%), 2 (10 – 100%), 3 (>100%) and LFW (Lysis from without).

Specie	Strain	Genotype	Score
Paenibacillus larvae	PI02-(93, 97, 100, 102, 110, 114, 116, 120)	ERIC I	LFW
Paenibacillus larvae	PI02-(127)	ERIC I	0
Paenibacillus larvae	PI02-(106, 122, 125, 126, 130, 131, 132, 134)	ERIC I	1
Paenibacillus larvae	PI02-(98, 99, 108, 111, 121, 128, 133, 135)	ERIC I	2
Paenibacillus larvae	PI02-(90, 95, 96, 101, 104, 105, 107, 109, 112, 113, 115, 117, 118, 119, 123, 124, 129, 136)	ERIC I	3

CHAPTER 4: COMPLETE GENOME SEQUENCES OF TWO *PAENIBACILLUS LARVAE* PHAGES

Abstract

Paenibacillus larvae is a Gram-positive bacterium that causes American Foulbrood disease (AFB), a serious concern in beekeeping. Here, are described two new *P. larvae* phages genomes from vB_PlaS_API46 (API46) and vB_PlaS_API59 (API59). API46 encodes 96 coding sequences (CDSs), while API59 encodes 62 CDSs in their 59,195 bp and 37,176 bp dsDNA genomes respectively. Both phages seem to have a siphovirus morphotype and were isolated from hive dust. The presence of lysogenic genes confirms than both are temperate phages (prophages).

4.1 Introduction

American Foulbrood disease (AFB) is a bacterial disease caused by Gram-positive *Paenibacillus larvae*. This bacterium is worldwide-distributed, which represent a severe problem to beekeeping sustainability and the crops pollination (Genersch, 2010a).

In order to restrict economic and ecological losses, new solutions to treat and prevent such infections are urgent, as antibiotics are inefficient and the burning of hives and material is mandatory (Genersch, 2010a; Miyagi et al., 2000). Currently, some studies suggests bacteriophages (phages) and their derivates proteins as feasible, effective and promising solutions (Beims et al., 2015; Brady et al., 2017; Ghorbani-Nezami et al., 2015; LeBlanc et al., 2015; A. Oliveira et al., 2015; Yost et al., 2016).

To date, 51 *P. larvae* phage genome sequences were whole sequenced and characterized (Beims et al., 2020; Bozdeveci et al., 2021; A. Oliveira et al., 2013; Ribeiro, Melo, et al., 2019; Stamereilers et al., 2018). Taxonomically, most of them were included in the *Siphoviridae* family (n=49). Only two belong to the *Podoviridae* family, vB_PlaP_API480 (Ribeiro, Melo, et al., 2019) and vB_PlaP_SV21 (Bozdeveci et al., 2021). The isolation and genomic characterization of two new siphovirus infecting *P. larvae* is reported here.

4.2 Material and methods

4.2.1 Bacterial strains: isolation and cultivation conditions

In this study, a total of 110 *P. larvae* strains were used (**Table 12**). From these, 67 were previously reported (Ribeiro, Melo, et al., 2019): 60 field strains (PI01-(01, 03, 07, 07b2, 13, 14, 18); PI02-(21, 23, 27, 30b, 31, 33–37, 45–81, 83–87); PI03-28) and seven reference strains: LMG 9820, CCUG 48972, CCUG 48973, LMG 15974, LMG 16252, LMG 16247 and LMG 16250. Additionally, 43 *P. larvae* strains PI02-(90, 93, 95–102, 104–136) newly isolated in the scope of this work, in September 2019 were used.

These strains were cultivated in MYPGP broth or agar (10 $g.L^{-1}$ Mueller-Hinton Broth (Oxoid); 15 $g.L^{-1}$ yeast extract (Oxoid); 3 $g.L^{-1}$ de K2HPO4 (LabKem); 1 $g.L^{-1}$ de Sodium-pyruvate (Fisher); 2% glucose (Ameresco) and 17 $g.L^{-1}$ agar (VWR) and incubated at 37 °C under 5% CO_2 overnight (O/N).

P. larvae isolation was performed as described by Genersch & Otten, (2003). Briefly, brood was from 82 hives: 53 from Bragança and 29 from the southern Portugal were analysed, using swabs to collect visually infected larvae material or grafting larvae in case of no visible signs. Samples were emulsified in 500 µL sterile water, heated at 90 °C, 6 minutes and sewed in MYPGP agar. After incubation for 3 to 6 days at 37 °C, 5% CO_2, single colonies were propagated in MYPGP agar and stored at -80 °C with 20% glycerol.

4.2.2 16S-PCR identification of *P. larvae* and rep-PCR analysis

For the newly isolated strains, bacterial DNA was purified from using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo) and amplified using Kapa *Taq* (Kapa Biosystems) according to the manufacturer's instructions.

The PCR primer sequences and conditions used for *P. larvae* identification (**Table 11**) were based on the *P. larvae* 16S rRNA gene (Dobbelaere et al., 2001). Positive results revealed a 1,106 bp band in a 1% (w/v) agarose gel under UV light.

The Enterobacterial Repetitive Intergenic Consensus (ERIC) genotyping of the previously identified *P. larvae* was accomplished through genomic fingerprinting as reported in Genersch & Otten, (2003) (primers and conditions are detailed in **Table 11**). ERIC patterns were visualised in a 2% agarose gel under UV light. LMG 9820, CCUG 48972, LMG 15974 and LMG 16247 were used as standard for ERIC I, II, III and IV profiles, respectively.

Table 11: PCR conditions for 16S-PCR of *P. larvae* identification and rep-PCR genotyping.

Target gene	Sequence (5′-3′)	PCR conditions
16S rRNA	Fw: CTTGTGTTTCTTTCGGGAGACGCC Rev: TCTTAGAGTGCCCACCTCTGCG	10 minutes 95 °C 15 seconds 95 °C; 15 seconds 60 °C; 15 seconds 72 °C (30 cycles) 3 minutes 72 °C
rep-PCR	ERIC2: AAGTAAGTGACTGGGGTGAGC ERIC1R: ATGTAAGCTCCTGGGGATTCA	3 minutes 95 °C 45 seconds 95 °C; 30 seconds 45 °C; 3 minutes 72 °C (30 cycles) 10 minutes 72 °C

4.2.3 Bacteriophages isolation, production and lytic spectra determination

Soil samples from hive surrounding dust collected in southern Portugal was used for phage isolation. For that, soil samples were suspended in MYPGP (2x) (1:10 w/v) and then mixed with groups of five bacterial strains pre-cultured O/N in MYPGP broth (37 °C, 5% CO_2). After another O/N incubation, the suspensions, centrifuged (10 minutes, 9000 *×g*, 4 °C) and the supernatant filtered-sterilized with 0.22 µm PES membranes (GE Healthcare). To confirm the presence of phages, 10 µL were spotted – "spot test" on the respective bacterial lawn. For lawns' preparation, 100 µl of the freshly grown strain was mixed with 3 mL 0.4% MYPGP agar and poured into agar plates. The presence of bacterial inhibition areas was indicative of host susceptibility to the phage. After O/N incubation at 37 °C, 5% CO_2 bacterial inhibition zones were picked and propagated over host bacterial lawns using sterile paper stripes (D. Pires et al., 2011). After a subsequent incubation, phages were isolated from a single phage plaque to obtain clones of confluent plaques. A volume of 2 mL SM buffer (5.8 $g.L^{-1}$ NaCl (PanReac); 2 $g.L^{-1}$ $MgSO_4.7H_2O$ (VWR); 50 $mL.L^{-1}$ 1 M Tris-HCl pH 7.5 (VWR)) was added to plates, and afterwards, the floating liquid, together with the soft-agar were collected, centrifuged (10 minutes, 9000 *×g*, 4 °C) and filtered-sterilized through 0.22 µm PES membranes. Phages were stored at 4 °C until use.

For phages propagation, 10 µL of the each stored phage suspension were spread respectively on its hosts bacterial lawns (propagating host (PI02-46 and PI02-59)). Plates were incubated O/N at 37 °C with 5% CO_2 and treated as described above for phage isolation, until a filter-sterilized high-titre phage suspension was obtained (stored at 4 °C).

The lytic activity of the isolated phages vB_PlaS_API46 (API46) and vB_PlaS_API59 (API59) was tested against the 110 *P. larvae* strains through spot test, as described above for phage isolation.

4.2.4 Electron microscopy analysis

Phage particles were collected by centrifugation (1 hour, 25000 *×g*, 4 °C) in a Beckman J2-21 centrifuge with a JA18.1 fixed rotor. The sediment was washed twice in tap water prior to centrifugation as above. Phages were deposited on copper grids with a carbon-coated Formvar film grid, stained with 2% uranyl acetate (pH 4.0) and examined using Jeol JEM 1400 transmission electron microscope (Tokyo, Japan).

4.2.5 DNA isolation, genome sequencing and annotation

P. larvae API46 and API59 phages genomic DNA were isolated using the phenol-chloroform-isoamyl alcohol method essentially as described in Melo et al., (2014). DNA samples were further used for library construction using the Illumina Nextera XT library preparation kit. The DNA libraries generated were sequenced in the Illumina MiSeq platform, using 250 bp paired-end sequencing reads. An automatic initial treatment was performed on raw sequence data, namely adapters and low-quality bases trimming. Demultiplexed reads were *de novo* assembled into a single contig using Geneious R9 (Biomatters, Newark, NJ, USA).

The assembled genomes were scanned through myRAST to search for coding regions (Aziz et al., 2008) and tRNAscan-SE to search for tRNAs (Schattner et al., 2005). To search for function, each DNA coding sequence (CDS) were analysed through BLASTp (default and with organism select to Tailed phages (tax id: 28883) simultaneously) (Altschul et al., 1990), as well in Conserved Domains-Search Tool for looking to conserver domains in Pfam database (Finn et al., 2014) with E-value threshold of 1×10^{-5} and HHpred (Soding et al., 2005) to search for similarities. Identified proteins were also analysed with TMHMM (Käll & Sonnhammer, 2002), Phobius (Käll, Krogh, & Sonnhammer, 2007) and SignalP to predict transmembrane domains and signal peptide cleavage sites (Petersen et al., 2011). Putative promoter regions were checked using PromoterHunter from phiSITE (Klucar et al., 2010) and were further manually verified. ARNold was used to predict factor-independent terminators (Naville et al., 2011) and the energy was calculated using Mfold (Zuker, 2003). The total genome or proteome were checked for antibiotic resistance genes through the ResFinder (Zankari et al., 2012) and the Resistance Gene Identifier (RGI) of CARD (The Comprehensive Antibiotic Resistance Database) (https://card.mcmaster.ca/analyze/rgi).

4.2.6 Comparative genomic analysis

To determine the relationship of the *P. larvae* API46 and API59 within all *P. larvae* phages (complete genome sequences deposited at GenBank as of December 2021 (n=51)), both whole phage genomes were compared and alignment through BLASTn, the analysis verified the E-value, homology and coverage parameters as previously described (H. Oliveira et al., 2019). The most similar phage (>90% identity) allowed us to infer the cluster of phages to which these new phages will belong.

4.3 Results

4.3.1 Phage isolation and host range

Two phages were isolated, API46 and API59, and their lytic spectra revealed that, API46 and API59 were able to infect 34 % and 62% of all field isolates, respectively (**Table 12**), all from ERIC I genotype, including the 43 newly isolated *P. larvae* strains (Supplementary Figure S25).

The opposite tendency was verified in the reference strains, from ERIC I to IV genotypes, API46 infected 71% and at least one strain of each genotype and API59 lysed 43%, but only two of the genotypes: ERIC I and ERIC II.

Table 12: Lytic spectra of API46 and API59, have positive activity (marked a green) and absence of activity (marked as orange) in 110 strains (103 field strains and 7 reference strains highlight in the gray box).

#Host	Strains	ERIC	API46	API59	#Host	Strains	ERIC	API46	API59	#Host	Strains	ERIC	API46	API59
1	1	I	+	+	57	L	I	+	+	100	B13260	I	-	+
3	3	I	+	+	58	87-6	I	+	+	101	B13263	I	-	-
7	7	I	+	+	59	SDA PIC/16	I	+	+	102	B13285	I	-	+
8	7b2	I	+	+	60	SDA-RO8	I	+	+	104	B13307	I	-	+
13	13	I	+	+	61	SDA P	I	+	+	105	B13317	I	+	+
14	14	I	+	+	62	Q	I	-	-	106	B13340	I	-	+
18	18	I	+	+	63	R	I	+	+	107	B13342	I	-	-
21	21	I	-	-	64	S	I	-	-	108	B13354	I	-	+
23	23	I	+	+	65	V	I	+	-	109	B13360	I	+	+
27	27	I	+	+	66	W	I	-	-	110	B13521	I	-	+
28	28	I	+	+	67	X	I	+	+	111	B13555	I	-	+
30	30b	I	-	-	68	Y	I	-	+	112	B13639	I	-	-
31	31	I	-	+	69	B12048	I	-	+	113	B13732	I	-	+
33	33	I	-	-	70	B11985	I	-	+	114	B13752	I	+	+
34	34	I	-	+	71	B12161	I	-	-	115	B13753	I	+	+
35	35	I	-	-	72	B12037	I	-	-	116	B13886	I	+	+
36	36	I	-	+	73	B12192	I	-	-	117	B14146	I	-	-
37	37 SLB	I	-	-	74	B12177	I	-	-	118	B14152	I	-	-
38	LMG 9820	I	+	+	75	B12277	I	-	-	119	B14198	I	-	-
39	CCUG 48972	II	+	+	76	B12174	I	-	-	120	B14246	I	-	-
40	CCUG 48973	II	+	+	77	B12430	I	+	-	121	B14247	I	-	-
41	LMG 15974	III	+	-	78	B12171	I	+	+	122	B14260	I	-	+
42	LMG 16252	III	-	-	79	B12186	I	+	+	123	B14343	I	-	+
43	LMG 16247	IV	+	-	80	B13119	I	-	-	124	B14773	I	-	-
44	LMG 16250	IV	-	-	81	B12248	I	-	-	125	B14980	I	-	-
45	PAT-17-16802	I	+	+	83	B12042	I	+	-	126	B15116	I	-	-
46	PAT-17-16945	I	+	+	84	B13117	I	-	+	127	B15162	I	-	+

47	PAT-17-18528	I	+	+	85	B12056	I	+	+	128	B15167	I	-	-
48	PAT-17-26190	I	-	-	86	PA13-156	I	-	+	129	B15168	I	+	+
49	PAT-17-19181	I	-	-	87	PA16-254	I	-	-	130	B15176	I	-	+
50	PAT-17-12200	I	-	-	90	24	I	-	-	131	B15179	I	-	+
51	PAT-14-26659	I	-	+	93	36B	I	-	-	132	B15180	I	-	+
52	PAT-17-12033	I	-	-	95	241B	I	-	+	133	B15183	I	-	+
53	260514	I	-	+	96	B13249	I	-	+	134	B15188	I	-	+
54	223314	I	-	-	97	B13251	I	-	+	135	B15189	I	-	+
55	J	I	+	+	98	B13253	I	+	+	136	B15190	I	-	+
56	K	I	+	+	99	B13255	I	-	+					

4.3.2 Phage morphology

Transmission electron microscopy (TEM) microphotographs (**Figure 24A**) revealed an intact API46 phage with a head of 73 × 44 nm and a long non-contractile tail of 115 × 12 nm. In the micrograph of phage API59 (**Figure 24B**) several fragments of the phage are present. The head is 104 × 70 nm and the non-contractile tail is 213 × 22 nm. Images suggest both phages as being siphovirus.

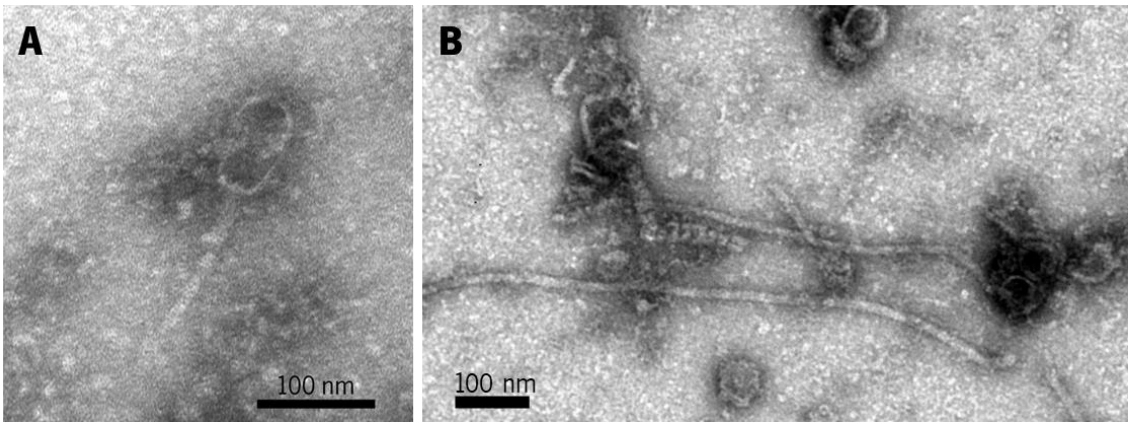

Figure 24: Transmission electron micrographs showing the virion particle morphology (stained with 2% uranyl acetate). Virion morphotype of: (A) API46 and (B) of API59. Scale bar: 100 nm.

4.3.3 Phage genomic and proteomic properties

4.3.3.1 General traits

Both phages API46 and API59 have genes related to structural, regulatory, DNA replication, lysis module and lysogenic functions. The API46 genome is a linear dsDNA molecule of 59,195 bp with 48.27% of GC content (**Table 13**). It encodes a total of 96 CDSs, of which 57 have hypothetical function (only 1 is unique on this phage) and 39 with an assigned function (**Table 14**). Most of the proteins (about 91.7%) are homologous to other phage proteins, while 7.3% are similar to host proteins. Only one protein is the unique hypothetical protein. Genes are tightly packed achieving 1.62 genes per 1,000 bp, with the genome being 89.59% coded. Furthermore, API46's genome has a translation of 67 proteins that start

on ATG codon (69.8%), 15 on GTG codon (15.6%), 13 on TTG codon (13.5%) and one on CTG codon (1%). Although no tRNA or antibiotic resistance genes were identified, eight promoters and eight factor-independent terminators were found, as well the integrase (gp30) and two antitoxins the antitoxin MazE (gp78) and antitoxin HicB (gp89).

API59 has a smaller genome (also linear dsDNA) of 37,176 bp with 42.13% of GC content (**Table 13**). It encodes a total of 62 CDSs, of which 28 have hypothetical function (none is unique on this phage) and 34 with an assigned function (**Table 15**). Around 98.4% of proteins are homologous to other phage proteins, while the remaining protein is similar to a *P. larvae* protein. Genes are tightly packed achieving 1.67 genes per 1,000 bp, with the genome being 91.26% coded. Furthermore, API59's genome has a translation of 52 proteins that start on ATG codon (83.9%), eight on TTG codon (12.9%) and two on GTG codon (3.2%). Although no tRNA genes were identified, six promoters and four factor-independent terminators were found, as well the recombinase (gp29), the metal β-lactamases (MBL) fold metallo-hydrolase (gp47) and the toxin-antitoxin (TA) system *hicAB*, with antitoxin HicB (gp59) and toxin HicA (gp60).

Table 13: *P. larvae* phages, genome characteristics, and closest published phages.

Phage designation	Host strain	Genome length (bp)	GC content (%)	No. of coding sequences	Closest phage deposited in GenBank	Morphology	Coding genome (%)
vB_PlaS_API46	PI02-46	59,195	48.27	96	Tripp	Siphovirus	89.95
vB_PlaS_API59	PI02-59	37,176	42.13	62	Eltigre	Siphovirus	91.26

4.3.3.2 Comparative analysis

The BLASTn analysis showed that API46 have higher homology to phage Tripp (E-value 0.0; Coverage 89%, Identity 99.53%), which belongs to Halcyone cluster. However, the alignment of API59 indicated Eltigre phage as the most homologous (E-value 0.0; Coverage 79%, Identity 99.03%), from cluster Fern. The high similarities between API46, API59 and the *P. larvae* phages that resulted BLASTn analysis is indicative that these phages are proximal and have a close relationship.

Table 14: API46 genome annotation and BLASTp or HHpred homology. CDSs unique to API46 are in white; CDSs homologue to bacteria are coloured in light grey; CDSs homologue to phages are coloured in dark grey; proteins with an identified function are highlighted in bold.

gp	Nt Initial	Nt Final	No. Nt	Strand	No. AA	Product	BLASTp/HHpred	E-value
1	772	951	180	+	59	Hypothetical protein	Hypothetical protein TRIPP_2 [Paenibacillus phage Tripp]	2,00E-32
2	1349	3115	1767	+	588	**Terminase large subunit**	Terminase large subunit [Paenibacillus phage Tripp]	0.0
3	3256	4638	1383	+	460	**Portal protein**	Portal protein [Paenibacillus phage Tripp]	0.0
4	4635	5495	861	+	286	**Minor capsid protein**	Minor capsid protein [Paenibacillus phage C7Cdelta]	0.0
5	5470	5583	114	+	37	Hypothetical protein	Hypothetical protein TRIPP_6 [Paenibacillus phage Tripp]	7,00E-18
6	5586	6221	636	+	211	**Scaffold protein**	Hypothetical protein [Paenibacillus larvae]	2,00E-149
7	6278	7228	951	+	316	**Major capsid protein**	Major capsid protein [Paenibacillus phage Tripp]	0.0
8	7243	7626	384	+	127	Hypothetical protein	Hypothetical protein TRIPP_9 [Paenibacillus phage Tripp]	8,00E-91
9	7627	7959	333	+	110	Hypothetical protein	Hypothetical protein TRIPP_10 [Paenibacillus phage Tripp]	2,00E-76
10	7956	8381	426	+	141	Hypothetical protein	Hypothetical protein TRIPP_11 [Paenibacillus phage Tripp]	7,00E-101
11	8378	8752	375	+	124	**Minor capsid protein**	Minor capsid protein [Paenibacillus larvae]	2,00E-84
12	8766	9314	549	+	182	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	4,00E-124
13	9366	9743	378	+	125	**Tail assembly protein**	Tail assembly protein [Paenibacillus phage C7Cdelta]	7,00E-85
14	9866	10093	228	+	75	**Tail assembly protein**	Tail assembly protein [Paenibacillus phage Tripp]	3,00E-49
15	10121	13009	2889	+	962	**Tail tape measure protein**	Tail tape measure protein [Paenibacillus phage Tripp]	0.0
16	13011	14468	1458	+	485	**Tail protein**	Tail protein [Paenibacillus phage Tripp]	0.0
17	14515	16791	2277	+	758	**Tail protein**	Tail protein [Paenibacillus phage Tripp]	0.0
18	16776	17225	450	+	149	Hypothetical protein	Hypothetical protein TRIPP_19 [Paenibacillus phage Tripp]	1,00E-101
19	17213	17629	417	+	138	**Holin**	Holin [Paenibacillus phage Ash]	1,00E-87
20	17622	18491	870	+	289	**N-acetylmuramoyl-L-alanine amidase (endolysin)**	N-acetylmuramoyl-L-alanine amidase [Paenibacillus phage Tripp]	0.0
21	18652	18879	228	+	75	Hypothetical protein	Hypothetical protein TRIPP_22 [Paenibacillus phage Tripp]	3,00E-48
22	18844	19212	369	-	122	**Transposase**	Transposase [Paenibacillus phage Tripp]	1,00E-85
23	19233	19370	138	-	45	**Transposase**	Transposase [Paenibacillus phage Tripp]	2,00E-22
24	19371	19493	123	+	40	Hypothetical protein	DDE-type integrase/transposase/recombinase [Paenibacillus larvae]	9,00E-08

25	19643	19882	240	+	79	Hypothetical protein	Hypothetical protein TRIPP_26 [Paenibacillus phage Tripp]	5,00E-50
26	19938	20570	633	-	210	Hypothetical protein	Hypothetical protein TRIPP_27 [Paenibacillus phage Tripp]	1,00E-150
27	20606	21100	495	-	164	Hypothetical protein	DUF4352 domain-containing protein [Paenibacillus larvae]	9,00E-115
28	21268	21654	387	-	128	Hypothetical protein	Hypothetical protein TRIPP_30 [Paenibacillus phage Tripp]	6,00E-87
29	21753	21875	123	-	40	Hypothetical protein	Hypothetical protein TRIPP_31 [Paenibacillus phage Tripp]	7,00E-20
30	21939	23117	1179	-	392	**Integrase**	Site-specific integrase [Paenibacillus phage Tripp]	0.0
31	23130	23771	642	-	213	**SOS-response repressor and protease LexA**	SOS-response repressor and protease LexA [Paenibacillus phage phiIBB_P123]	4,00E-154
32	23946	24167	222	+	73	**Helix-turn-helix transcriptional regulator**	Helix-turn-helix transcriptional regulator [Paenibacillus phage Tripp]	2,00E-48
33	24191	24472	282	+	93	**Helix-turn-helix domain-containing protein**	Helix-turn-helix domain-containing protein [Paenibacillus phage Tripp]	2,00E-63
34	24585	24887	303	+	100	**Rha family regulatory protein**	Rha family regulatory protein [Paenibacillus phage Tripp]	2,00E-69
35	24928	25566	639	+	212	Hypothetical protein	Hypothetical protein TRIPP_38 [Paenibacillus phage Tripp]	2,00E-158
36	25588	26382	795	+	264	**Antirepressor KilAC domain-containing protein**	Antirepressor KilAC domain-containing protein [Paenibacillus phage Tripp]	0.0
37	26379	26630	252	+	83	Hypothetical protein	Hypothetical protein TRIPP_40 [Paenibacillus phage Tripp]	2,00E-53
38	26627	26800	174	+	57	Hypothetical protein	Hypothetical protein TRIPP_41 [Paenibacillus phage Tripp]	4,00E-34
39	26797	27099	303	+	100	Hypothetical protein	Hypothetical protein TRIPP_42 [Paenibacillus phage Tripp]	2,00E-67
40	27126	27452	327	+	108	Hypothetical protein	Hypothetical protein TRIPP_43 [Paenibacillus phage Tripp]	1,00E-74
41	27474	27800	327	+	108	**Helix-turn-helix domain-containing protein**	Helix-turn-helix domain-containing protein [Paenibacillus phage Tripp]	3,00E-75
42	27969	28217	249	+	82	Hypothetical protein	Hypothetical protein TRIPP_45 [Paenibacillus phage Tripp]	2,00E-56
43	28248	28613	366	+	121	Hypothetical protein	Hypothetical protein TRIPP_46 [Paenibacillus phage Tripp]	9,00E-84
44	28671	29042	372	+	123	Hypothetical protein	Hypothetical protein TRIPP_47 [Paenibacillus phage Tripp]	1,00E-88
45	29026	29394	369	+	122	Hypothetical protein	Hypothetical protein TRIPP_48 [Paenibacillus phage Tripp]	8,00E-88
46	29410	30261	852	+	283	Hypothetical protein	DUF3102 domain-containing protein [Paenibacillus phage Tripp]	0.0
47	30338	30862	525	+	174	Hypothetical protein	Hypothetical protein TRIPP_50 [Paenibacillus phage Tripp]	3,00E-125
48	30873	31145	273	+	90	Hypothetical protein	Hypothetical protein TRIPP_51 [Paenibacillus phage Tripp]	2,00E-62
49	31147	31917	771	+	256	**DNA replication protein**	DNA replication protein [Paenibacillus phage Tripp]	0.0
50	31914	33290	1377	+	458	**Replicative DNA helicase**	AAA family ATPase [Paenibacillus phage Tripp]	0.0
51	33303	34253	951	+	316	**DNA primase**	DNA primase / toprim domain-containing protein [Paenibacillus phage Tripp]	0.0

52	34306	34869	564	+	187	Hypothetical protein	Hypothetical protein TRIPP_55 [Paenibacillus phage Tripp]	5,00E-136
53	34986	35459	474	+	157	Hypothetical protein	Hypothetical protein TRIPP_56 [Paenibacillus phage Tripp]	9,00E-107
54	35546	36283	738	+	245	**Single-stranded DNA-binding protein**	Single-stranded DNA-binding protein [Paenibacillus phage Tripp]	0.0
55	36465	36680	216	+	71	Hypothetical protein	Hypothetical protein TRIPP_59 [Paenibacillus phage Tripp]	3,00E-47
56	36677	36796	120	+	39	Hypothetical protein	Hypothetical protein TRIPP_60 [Paenibacillus phage Tripp]	2,00E-19
57	36825	37238	414	+	137	Hypothetical protein	Hypothetical protein TRIPP_61 [Paenibacillus phage Tripp]	2,00E-98
58	37226	37435	210	+	69	Hypothetical protein	Hypothetical protein TRIPP_62 [Paenibacillus phage Tripp]	3,00E-44
59	37446	37814	369	+	122	Hypothetical protein	Hypothetical protein TRIPP_63 [Paenibacillus phage Tripp]	9,00E-84
60	37807	38613	807	+	268	Hypothetical protein	Hypothetical protein TRIPP_64 [Paenibacillus phage Tripp]	0.0
61	38755	40410	1656	+	551	**DNA polymerase I**	DNA polymerase I [Paenibacillus phage Tripp]	0.0
62	40734	41900	1167	+	388	**DNA polymerase I**	DNA polymerase [Paenibacillus phage Tripp]	0.0
63	41901	42098	198	+	65	Hypothetical protein	Hypothetical protein TRIPP_68 [Paenibacillus phage Tripp]	2,00E-42
64	42095	42709	615	+	204	**DNA polymerase III alpha subunit**	3'-5' exonuclease [Paenibacillus phage Tripp]	2,00E-149
65	42853	43713	861	+	286	Hypothetical protein	Hypothetical protein ASH_62 [Paenibacillus phage Ash]	0.0
66	43913	44221	309	+	102	**Resolvase RuvC-like**	Resolvase [Paenibacillus phage C7Cdelta]	6,00E-71
67	44218	44391	174	+	57	Hypothetical protein	Hypothetical protein KMD17_gp64 [Paenibacillus phage C7Cdelta]	3,00E-34
68	44459	46675	2217	+	738	**Ribonucleotide diphosphate reductase alpha subunit**	Ribonucleotide diphosphate reductase alpha subunit [Paenibacillus phage C7Cdelta]	0.0
69	46689	47720	1032	+	343	**Ribonucleotide diphosphate reductase beta subunit**	Ribonucleotide diphosphate reductase beta subunit [Paenibacillus phage C7Cdelta]	0.0
70	47732	48256	525	+	174	**Deoxyuridine 5'-triphosphate nucleotidohydrolase**	Deoxyuridine 5'-triphosphate nucleotidohydrolase [Paenibacillus phage vB_PlaP_API480]	1,00E-83
71	48258	48392	135	+	44	Hypothetical protein	Hypothetical protein ERIC1_1c14510 [Paenibacillus larvae subsp. larvae DSM 25719]	2,00E-22
72	48442	48732	291	+	96	Hypothetical protein	Hypothetical protein KMD17_gp70 [Paenibacillus phage C7Cdelta]	2,00E-58
73	48735	49073	339	+	112	Hypothetical protein	Hypothetical protein KMD17_gp71 [Paenibacillus phage C7Cdelta]	6,00E-71
74	49073	49267	195	+	64	Hypothetical protein	Hypothetical protein [Paenibacillus larvae]	3,00E-36
75	49268	49594	327	+	108	Hypothetical protein	Hypothetical protein KMD17_gp72 [Paenibacillus phage C7Cdelta]	6,00E-73
76	49608	50021	414	+	137	Hypothetical protein	Hypothetical protein KMD17_gp73 [Paenibacillus phage C7Cdelta]	2,00E-79
77	50000	50230	231	+	76	Hypothetical protein	Hypothetical protein KMD17_gp74 [Paenibacillus phage C7Cdelta]	2,00E-41
78	50232	50549	318	+	105	**Antitoxin MazE**	Hypothetical protein ASH_75 [Paenibacillus phage Ash]	4,00E-69

79	50543	50716	174	+	57	Hypothetical protein	Hypothetical protein KMD18_gp77 [Paenibacillus phage Halcyone]	4,00E-27
80	50863	51006	144	+	47	Hypothetical protein	Hypothetical protein ASH_76 [Paenibacillus phage Ash]	3,00E-22
81	51003	51371	369	+	122	Hypothetical protein	Hypothetical protein KMD18_gp78 [Paenibacillus phage Halcyone]	1,00E-81
82	51598	52455	858	+	285	Hypothetical protein	Hypothetical protein LEY_78 [Paenibacillus phage Ley]	7,00E-150
83	52439	52999	561	+	186	**Deoxynucleoside monophosphate kinase**	Deoxynucleoside monophosphate kinase [Paenibacillus phage Ley]	4,00E-123
84	52956	53339	384	-	127	Hypothetical protein	Hypothetical protein TRIPP_81 [Paenibacillus phage Tripp]	3,00E-88
85	53466	54656	1191	+	396	DNA methyltransferase	DNA methyltransferase [Paenibacillus phage Tripp]	0.0
86	54644	55048	405	+	134	**RNA polymerase subunit sigma**	RNA polymerase subunit sigma [Paenibacillus phage Tripp]	1,00E-94
87	55038	55496	459	+	152	Hypothetical protein	Hypothetical protein TRIPP_84 [Paenibacillus phage Tripp]	7,00E-109
88	55683	55820	138	+	45	Hypothetical protein	Hypothetical protein TRIPP_85 [Paenibacillus phage Tripp]	7,00E-26
89	55886	56293	408	+	135	**HicB antitoxin**	Type II toxin-antitoxin system HicB family antitoxin [Paenibacillus phage Tripp]	1,00E-96
90	56468	56734	267	+	88	Hypothetical protein	Hypothetical protein TRIPP_87 [Paenibacillus phage Tripp]	1,00E-54
91	56731	57567	837	+	278	Hypothetical protein	Hypothetical protein TRIPP_88 [Paenibacillus phage Tripp]	0.0
92	57564	57677	114	+	37	Hypothetical protein	Hypothetical protein	
93	57910	58128	219	+	72	Hypothetical protein	Hypothetical protein TRIPP_89 [Paenibacillus phage Tripp]	1,00E-44
94	58118	58285	168	+	55	Hypothetical protein	Hypothetical protein TRIPP_90 [Paenibacillus phage Tripp]	3,00E-33
95	58287	58706	420	+	139	Hypothetical protein	Hypothetical protein KMD17_gp86 [Paenibacillus phage C7Cdelta]	8,00E-97
96	58696	59085	390	+	129	**Helix-turn-helix domain-containing protein**	Helix-turn-helix domain-containing protein [Paenibacillus phage Tripp]	7,00E-93

Table 15: API59 genome annotation and BLASTp or HHpred homology. CDSs homologue to bacteria are coloured in light grey; CDSs homologue to phages are coloured in dark grey; proteins with an identified function are highlighted in bold.

gp	Nt Initial	Nt Final	No. Nt	Strand	No. AA	Product	BLASTp/HHpred	E-value
1	50	364	315	+	104	**Terminase small subunit**	Terminase small subunit [Paenibacillus phage HB10c2]	1,00E-71
2	345	2069	1725	+	574	**Terminase large subunit**	Terminase large subunit [Paenibacillus phage phiIBB_P123]	0.0
3	2066	3319	1254	+	417	**Portal protein**	Portal protein [Paenibacillus phage Pagassa]	0.0
4	3303	4025	723	+	240	**Clp protease**	Clp protease [Paenibacillus phage Fern]	8E-180
5	4022	5152	1131	+	376	**Major capsid protein**	Major capsid protein [Paenibacillus phage phiIBB_P123]	0.0
6	5130	5303	174	+	57	Hypothetical protein	Hypothetical protein IBBPI23_06 [Paenibacillus phage phiIBB_P123]	2,00E-30
7	5296	5559	264	+	87	**Head-tail connector complex protein**	Head-tail connector protein [Paenibacillus phage phiIBB_P123]	2E-57
8	5556	5873	318	+	105	**Head-tail connector complex protein**	Head closure protein [Paenibacillus phage phiIBB_P123]	9,00E-75
9	5873	6301	429	+	142	Hypothetical protein	Hypothetical protein IBBPI23_09 [Paenibacillus phage phiIBB_P123]	3,00E-99
10	6348	6680	333	+	110	**DUF3168 domain-containing protein**	DUF3168 domain-containing protein [Paenibacillus phage phiIBB_P123]	1,00E-74
11	6682	7119	438	+	145	**Major tail protein**	Major tail protein, TP901-1 family [Paenibacillus phage phiIBB_P123]	6,00E-103
12	7116	7454	339	+	112	**Tail assembly protein**	Tail assembly protein [Paenibacillus phage HB10c2]	3,00E-76
13	7481	7735	255	+	84	**Tail assembly protein**	Tail assembly protein [Paenibacillus phage phiIBB_P123]	3,00E-55
14	7795	10431	2637	+	878	**Tail tape measure protein**	Tail tape measure protein [Paenibacillus phage phiIBB_P123]	0.0
15	10413	11279	867	+	288	**Tail protein**	Tail protein [Paenibacillus phage phiIBB_P123]	0.0
16	11282	12400	1119	+	372	**Tail protein**	Tail protein [Paenibacillus phage phiIBB_P123]	0.0
17	12397	13479	1083	+	360	**Tail protein**	Tail protein [Paenibacillus phage Xenia]	0.0
18	13480	13827	348	+	115	Hypothetical protein	Hypothetical protein XENIA_18 [Paenibacillus phage Xenia]	8,00E-79
19	13824	13985	162	+	53	Hypothetical protein	Hypothetical protein AVV25_gp19 [Paenibacillus phage Diva]	2,00E-34
20	13963	14205	243	+	80	**Holin**	Bacteriocin UviB precursor [Paenibacillus phage HB10c2]	1E-52
21	14205	14876	672	+	223	**N-acetylmuramoyl-L-alanine amidase (endolysin)**	N-acetylmuramoyl-L-alanine amidase [Paenibacillus phage Xenia]	3,00E-170
22	14886	15128	243	+	80	**Holin**	Putative holin [Paenibacillus phage Yyerffej]	7,00E-48
23	15374	15613	240	-	79	Hypothetical protein	Hypothetical protein AXJ12_gp23 [Paenibacillus phage Rani]	2,00E-48
24	15691	16485	795	-	264	Hypothetical protein	Hypothetical protein AVV26_gp24 [Paenibacillus phage HB10c2]	0.0
25	16632	16892	261	-	86	Hypothetical protein	Hypothetical protein IBBPI23_27 [Paenibacillus phage phiIBB_P123]	5,00E-38
26	17514	17660	147	+	48	Hypothetical protein	Hypothetical protein IBBPI23_27B [Paenibacillus phage phiIBB_P123]	4,00E-21

27	17684	17842	159	+	52	Hypothetical protein	Hypothetical protein IBBPI23_28 [Paenibacillus phage phiIBB_P123]	3,00E-26
28	17945	18187	243	+	80	Hypothetical protein	Hypothetical protein IBBPI23_29 [Paenibacillus phage phiIBB_P123]	1,00E-52
29	18283	19887	1605	-	534	**Recombinase**	Recombination protein [Paenibacillus phage phiIBB_P123]	0.0
30	19890	20573	684	-	227	**SOS-response repressor and protease LexA**	SOS-response repressor and protease LexA [Paenibacillus phage phiIBB_P123]	1E-170
31	20686	20931	246	+	81	Hypothetical protein	Hypothetical protein IBBPI23_32 [Paenibacillus phage phiIBB_P123]	5,00E-56
32	21092	21304	213	+	70	Hypothetical protein	Hypothetical protein IBBPI23_33 [Paenibacillus phage phiIBB_P123]	4,00E-48
33	21346	21615	270	+	89	Hypothetical protein	Hypothetical protein IBBPI23_34 [Paenibacillus phage phiIBB_P123]	4,00E-60
34	21636	21839	204	+	67	Hypothetical protein	Hypothetical protein IBBPI23_35 [Paenibacillus phage phiIBB_P123]	3,00E-43
35	21846	22223	378	+	125	Hypothetical protein	Hypothetical protein IBBPI23_36 [Paenibacillus phage phiIBB_P123]	1,00E-88
36	22220	22450	231	+	76	Hypothetical protein	Hypothetical protein IBBPI23_37 [Paenibacillus phage phiIBB_P123]	4E-50
37	22434	22607	174	+	57	Hypothetical protein	Hypothetical protein IBBPI23_38 [Paenibacillus phage phiIBB_P123]	5,00E-35
38	22612	23364	753	+	250	**Antirepressor KilAC domain-containing protein**	Antirepressor KilAC domain-containing protein [Paenibacillus phage Harrison]	0.0
39	23361	23864	504	+	167	Hypothetical protein	Hypothetical protein HWB48_gp40 [Paenibacillus phage Likha]	7,00E-116
40	23891	24085	195	+	64	Hypothetical protein	Hypothetical protein ERICV_05098 [Paenibacillus phage phiERICV]	7,00E-38
41	24078	24449	372	+	123	Hypothetical protein	Hypothetical protein AVV24_gp49 [Bacteriophage Lily]	8,00E-61
42	24465	24725	261	+	86	Hypothetical protein	Hypothetical protein XENIA_53 [Paenibacillus phage Xenia]	1,00E-44
43	24730	25020	291	+	96	Hypothetical protein	Hypothetical protein IBBPI23_43 [Paenibacillus phage phiIBB_P123]	2,00E-63
44	25004	26533	1530	+	509	**DNA repair protein**	AAA family ATPase [Paenibacillus phage phiIBB_P123]	0.0
45	26530	26727	198	+	65	Hypothetical protein	Hypothetical protein IBBPI23_45 [Paenibacillus phage phiIBB_P123]	2,00E-42
46	26724	27644	921	+	306	**DNA repair protein**	DNA recombinational protein RecT [Paenibacillus phage Fern]	0.0
47	27657	28415	759	+	252	**MBL fold metallo-hydrolase**	MBL fold metallo-hydrolase [Paenibacillus phage phiIBB_P123]	0.0
48	28412	28726	315	+	104	Hypothetical protein	Hypothetical protein IBBPI23_48 [Paenibacillus phage phiIBB_P123]	8,00E-73
49	28741	29571	831	+	276	Hypothetical protein	Primosome component-like protein [Paenibacillus phage phiIBB_P123]	0.0
50	29531	30859	1329	+	442	**Replicative DNA helicase**	Replicative DNA helicase [Paenibacillus phage phiIBB_P123]	0.0
51	30856	31167	312	+	103	Hypothetical protein	Hypothetical protein HWB43_gp61 [Paenibacillus phage BN12]	5E-72
52	31178	32230	1053	+	350	**DNA-cytosine methyltransferase**	DNA-cytosine methyltransferase [Paenibacillus phage Rani]	0.0
53	32227	32532	306	+	101	**MazG-like family protein**	MazG-like family protein [Paenibacillus larvae]	7,00E-68
54	32570	33022	453	+	150	**YopX protein**	Protein YopX [Paenibacillus phage BN12]	7,00E-111
55	33015	33230	216	+	71	Hypothetical protein	Hypothetical protein AVV26_gp49 [Paenibacillus phage HB10c2]	3,00E-48

56	33194	33784	591	+	196	**Resolvase RuvC-like**	Resolvase RuvC-like [Paenibacillus phage Rani]	3,00E-145
57	33943	34401	459	+	152	Hypothetical protein	Hypothetical protein AVV26_gp51 [Paenibacillus phage HB10c2]	1,00E-106
58	34452	34892	441	+	146	**ArpU-like transcriptional regulator**	ArpU-like transcriptional regulator [Paenibacillus phage phiIBB_P123]	3,00E-105
59	35032	35454	423	-	140	**HicB antitoxin**	Type II toxin-antitoxin system HicB family antitoxin [Paenibacillus larvae]	3,00E-100
60	35516	35731	216	-	71	**HicA toxin**	Type II toxin-antitoxin system HicA family toxin [Paenibacillus phage HB10c2]	1,00E-46
61	36521	36787	267	+	88	**Transglycosylase**	Transglycosylase [Paenibacillus phage HB10c2]	2,00E-58
62	36784	37122	339	+	112	**HNH endonuclease**	HNH endonuclease [Paenibacillus phage Xenia]	7,00E-80

4.4 Discussion

The isolation and genome characterization of API46 and API59, revealed that both are very similar to the previously reported temperate phages (Stamereilers et al., 2018). Their genes are common among *P. larvae* phage population, and are related to the various functions structural, DNA replication, regulation, host lysis and lysogenic lifecycle. However, their potential as therapeutic agents, to use against AFB needs to be evaluated in detail, as both seem to be temperate phages: API46 encodes an integrase (gp30) and API59 a recombinase (gp29) gene, respectively.

Due to its better lytic spectrum and smaller genome, API59 is more promissing than API46. The small genome makes it a better candidate to be genetically manipulated through synthetic and molecular biology, that could be needed to avoid lysogeny. It would be desirable to remove the recombinase gene (gp29) and increase their safety by eliminating the TA-system *hicAB* (antitoxin HicB (gp59) and toxin HicA (gp60)), the toxin encoded by YopX (gp54) and the MBL fold metallo-hydrolase (gp47), associated to hydrolyses the β-lactam antibiotics class B. On the other hand, the application of phage cocktails to control *P. larvae* delaying resistances can be pondered, but for that, phage receptors need to be investigated.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., ... Zagnitko, O. (2008). The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics*, *9*(1), 75. https://doi.org/10.1186/1471-2164-9-75

Beims, H., Bunk, B., Erler, S., Mohr, K. I., Spröer, C., Pradella, S., ... Steinert, M. (2020). Discovery of Paenibacillus larvae ERIC V: Phenotypic and genomic comparison to genotypes ERIC I-IV reveal different inventories of virulence factors which correlate with epidemiological prevalences of American Foulbrood. *International Journal of Medical Microbiology*, *310*(2), 151394. https://doi.org/10.1016/j.ijmm.2020.151394

Beims, H., Wittmann, J., Bunk, B., Spröer, C., Rohde, C., Günther, G., ... Steinert, M. (2015). Paenibacillus larvae-Directed bacteriophage HB10c2 and its application in American foulbrood-affected honey bee larvae. *Applied and Environmental Microbiology*, *81*(16), 5411–5419. https://doi.org/10.1128/AEM.00804-15

Bozdeveci, A., Akpınar, R., & Karaoğlu, Ş. A. (2021). Isolation, characterization, and comparative genomic analysis of vB_PlaP_SV21, new bacteriophage of Paenibacillus larvae. *Virus Research*, *305*(September), 198571. https://doi.org/10.1016/j.virusres.2021.198571

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of Paenibacillus larvae in honeybee hives. *Journal of Invertebrate Pathology*, *150*(September), 94–100. https://doi.org/10.1016/j.jip.2017.09.010

Dobbelaere, W., de Graaf, D. C., & Peeters, J. E. (2001). Development of a fast and reliable diagnostic method for American foulbrood disease (Paenibacillus larvae subsp. larvae) using a 16S rRNA gene based PCR. *Apidologie*, *32*(4), 363–370. https://doi.org/10.1051/apido:2001136

Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., ... Punta, M. (2014). Pfam: The protein families database. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkt1223

Genersch, E. (2010). American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. *Journal of Invertebrate Pathology*, *103*(SUPPL. 1), S10–S19. https://doi.org/10.1016/j.jip.2009.06.015

Genersch, E., & Otten, C. (2003). The use of repetitive element PCR fingerprinting (rep-PCR) for genetic subtyping of German field isolates of Paenibacillus larvae subsp. larvae. *Apidologie*, *34*(3), 195–206. https://doi.org/10.1051/apido:2003025

Ghorbani-Nezami, S., LeBlanc, L., Yost, D. G., & Amy, P. S. (2015). Phage Therapy is Effective in Protecting Honeybee Larvae from American Foulbrood Disease. *Journal of Insect Science*, *15*(1), 84. https://doi.org/10.1093/jisesa/iev051

Käll, L., Krogh, A., & Sonnhammer, E. L. L. (2007). Advantages of combined transmembrane topology and signal peptide prediction-the Phobius web server. *Nucleic Acids Research*, *35*(SUPPL.2), 429–432. https://doi.org/10.1093/nar/gkm256

Käll, L., & Sonnhammer, E. L. . (2002). Reliability of transmembrane predictions in whole-genome data. *FEBS Letters*, *532*(3), 415–418. https://doi.org/10.1016/S0014-5793(02)03730-4

Klucar, L., Stano, M., & Hajduk, M. (2010). phiSITE: database of gene regulation in bacteriophages. *Nucleic Acids Research*, *38*(Database), D366–D370. https://doi.org/10.1093/nar/gkp911

LeBlanc, L., Nezami, S., Yost, D., Tsourkas, P., & Amy, P. S. (2015). Isolation and characterization of a novel phage lysin

active against Paenibacillus larvae , a honeybee pathogen. *Bacteriophage*, *5*(4), e1080787. https://doi.org/10.1080/21597081.2015.1080787

Melo, L. D. R., Sillankorva, S., Ackermann, H.-W., Kropinski, A. M., Azeredo, J., & Cerca, N. (2014). Isolation and characterization of a new Staphylococcus epidermidis broad-spectrum bacteriophage. *Journal of General Virology*, *95*(Pt_2), 506–515. https://doi.org/10.1099/vir.0.060590-0

Miyagi, T., Peng, C. Y. S., Chuang, R. Y., Mussen, E. C., Spivak, M. S., & Doi, R. H. (2000). Verification of oxytetracycline-resistant American foulbrood pathogen Paenibacillus larvae in the United States. *Journal of Invertebrate Pathology*, *75*(1), 95–96. https://doi.org/10.1006/jipa.1999.4888

Naville, M., Ghuillot-Gaudeffroy, A., Marchais, A., & Gautheret, D. (2011). ARNold: A web tool for the prediction of Rho-independent transcription terminators. *RNA Biology*, *8*(1), 11–13. https://doi.org/10.4161/rna.8.1.13346

Oliveira, A., Leite, M., Kluskens, L. D., Santos, S. B., Melo, L. D. R., & Azeredo, J. (2015). The First Paenibacillus larvae Bacteriophage Endolysin (PlyPl23) with High Potential to Control American Foulbrood. *PLOS ONE*, *10*(7), e0132095. https://doi.org/10.1371/journal.pone.0132095

Oliveira, A., Melo, L. D. R., Kropinski, A. M., & Azeredo, J. (2013). Complete Genome Sequence of the Broad-Host-Range Paenibacillus larvae Phage phiIBB_Pl23. *Genome Announcements*, *1*(5), e00438-13-e00438-13. https://doi.org/10.1128/genomeA.00438-13

Oliveira, H., Sampaio, M., Melo, L. D. R., Dias, O., Pope, W. H., Hatfull, G. F., & Azeredo, J. (2019). Staphylococci phages display vast genomic diversity and evolutionary relationships. *BMC Genomics*, *20*(1), 357. https://doi.org/10.1186/s12864-019-5647-8

Petersen, T. N., Brunak, S., von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods*, *8*(10), 785–786. https://doi.org/10.1038/nmeth.1701

Pires, D., Sillankorva, S., Faustino, A., & Azeredo, J. (2011). Use of newly isolated phages for control of Pseudomonas aeruginosa PAO1 and ATCC 10145 biofilms. *Research in Microbiology*, *162*(8), 798–806. https://doi.org/10.1016/j.resmic.2011.06.010

Ribeiro, H. G., Melo, L. D. R., Oliveira, H., Boon, M., Lavigne, R., Noben, J.-P., … Oliveira, A. (2019). Characterization of a new podovirus infecting Paenibacillus larvae. *Scientific Reports*, *9*(1), 20355. https://doi.org/10.1038/s41598-019-56699-y

Schattner, P., Brooks, A. N., & Lowe, T. M. (2005). The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs. *Nucleic Acids Research*, *33*(Web Server), W686–W689. https://doi.org/10.1093/nar/gki366

Soding, J., Biegert, A., & Lupas, A. N. (2005). The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Research*, *33*(Web Server), W244–W248. https://doi.org/10.1093/nar/gki408

Stamereilers, C., Fajardo, C., Walker, J., Mendez, K., Castro-Nallar, E., Grose, J., … Tsourkas, P. (2018). Genomic Analysis of 48 Paenibacillus larvae Bacteriophages. *Viruses*, *10*(7), 377. https://doi.org/10.3390/v10070377

Yost, D. G., Tsourkas, P., & Amy, P. S. (2016). Experimental bacteriophage treatment of honeybees (Apis mellifera) infected with Paenibacillus larvae, the causative agent of American foulbrood disease. *Bacteriophage*, *6*(1), e1122698. https://doi.org/10.1080/21597081.2015.1122698

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., … Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *Journal of Antimicrobial Chemotherapy*, *67*(11), 2640–2644. https://doi.org/10.1093/jac/dks261

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research*, *31*(13), 3406–3415. https://doi.org/10.1093/nar/gkg595

Supplementary information

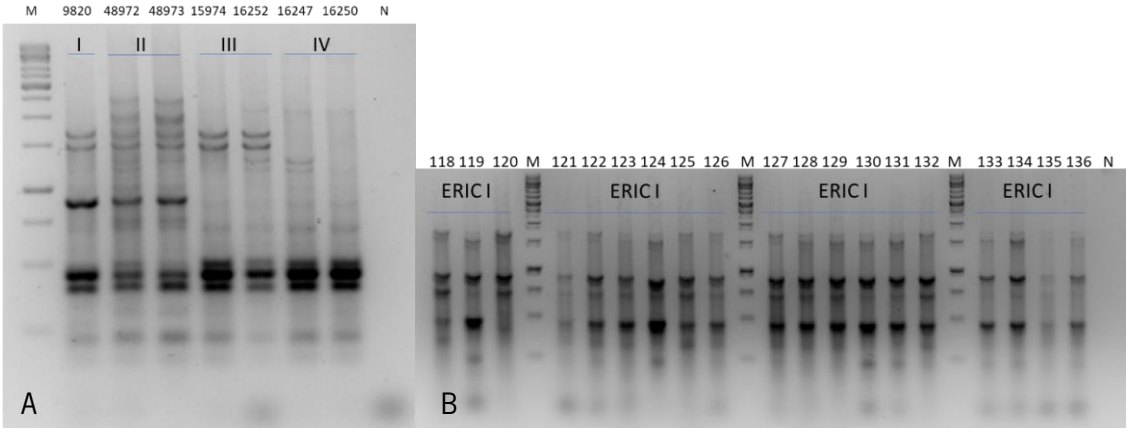

Figure S25: Agarose gel of *P. larvae* genotyping. (A) Standard genotyping using reference strains. (B) Genotype classification of some field strains present in Table 12. M: GeneRuler 1 kb DNA Ladder.

CHAPTER 5: *IN VIVO* ASSAYS IN HEALTHY HIVES AND IN LAB-REARED LARVAE

Abstract

American Foulbrood disease (AFB) is caused by the contagious bacteria, *Paenibacillus larvae*. Nowadays, the therapeutics solutions are missing, and phage therapy seems to be a good alternative to handle this disease. However, the protection of phages from harsh hive environment may be important.

In the present work, the biodistribution of vB_PlaP_API480 (API480) in experimental hives, either encapsulated (pH-responsive polymer Eudragit S100) or free were performed. The presence of phage in adult and larvae bee after providing phage formulations in bee feeders was firstly investigated. In both cases, after 24 hours, viable phage particles were recovered from larvae tissue, supporting this way of delivery. However, S100 formulation provided a larger number of infective phages to adult bees (crops and mid- hindguts) comparatively to free phage (an average of 186 PFU/bee and 86 PFU/bee, respectively). Then, the biosecurity and efficiency of suspensions of free phages, API480 and also vB_PlaS_CEB16 (CEB16) on lab-reared larvae were tested. No negative impact in larvae was reported after the ingestion of both phages. Furthermore, the time-lapsed treatment of *P. larvae*-infected larvae with phage API480 followed by CEB16 was able to reduce bacterial load by 2.0 Log CFU/larva, in 47% of the treated insects. When API480 was administered individually, at once or time-lapsed, no reduction of *P. larvae* was observed in highly infected larvae.

Overall, these assays together suggest that the application of encapsulated phages seems to be an effective way to provide viable phages to larvae after administration to adult bees, and that time-lapsed phage administration with different phages may improve the prevention and control of AFB.

5.1 Introduction

One of the many diseases affecting honeybees, *Apis mellifera* is American Foulbrood disease (AFB), which is caused by the Gram-positive bacterium *Paenibacillus larvae* (Genersch, 2010a). This disease directly affects the brood, as adult bees are only the vector of transmission by feeding larvae with contaminated food (Genersch et al., 2005; Woodrow, 1942; Woodrow & Holst, 1942). *P. larvae*, produce extremely resistant spores that when arrive in midgut larvae germinate, proliferate and invade tissues by breaking the epithelium, causing the larvae death (Bassi et al., 2015; Genersch, 2006; Genersch et al., 2005; Yue et al., 2008).

Nowadays, beekeepers try to control AFB, with sodium hydroxide disinfections, selecting honeybees with more skills to perform a better hygienic behaviour (Genersch, 2010a), artificial swarming, or applying commensal bacteria (Daisley et al., 2020) and natural products like propolis or essential oils to prevent the infection or decrease the symptoms (Genersch, 2017; Pellegrini, Alonso-Salces, et al., 2017), but the burning of the hives and the materials is mandatory (Genersch, 2010a). Moreover, the treatment with antibiotics is forbidden in Europe (EC regulation 2377/90 and further amendments (European Parliament and the Council of the European Union, 2010)) to avoid antibiotic resistance spread and consumption of antibiotic residues in hive-derived products (Genersch, 2010a; Miyagi et al., 2000; Wu et al., 2005).

Considering the urgency of new therapeutic solutions, the introduction of bacteriophage (phage) treatments seems promising. Phages are highly specific virus that exclusively infect and lyse their bacteria hosts (Abedon et al., 2011). The use of phage therapy is currently a viable alternative to treat and prevent bacterial infections either on humans, animals, plants or surfaces (Abedon et al., 2011; Kutateladze & Adamia, 2010; Sagona, Grigonyte, MacDonald, & Jaramillo, 2016; Sulakvelidze et al., 2001; Summers et al., 2012). Several studies in different applications were already reported, such as those in agriculture (Adriaenssens et al., 2012; Y. J. Bae et al., 2012), food safety (Bigwood, Hudson, Billington, Carey-Smith, & Heinemann, 2008; Leverentz et al., 2003; Spricigo, Bardina, Cortés, & Llagostera, 2013), or veterinary medicine (C. Carvalho, Costa, Silva, & Oliveira, 2017; C. M. Carvalho et al., 2010; Hawkins, Harper, Burch, Änggård, & Soothill, 2010; Oliveira, Sereno, & Azeredo, 2010).

With regard to beekeeping, some studies have evaluated the efficacy of phage therapies in lab-reared bee larvae (Beims et al., 2015; Ghorbani-Nezami et al., 2015; Yost et al., 2016) and in hives (Brady et al., 2017).

Later, in 2019, a study investigating the potential of the phage oral administration to adult bees in AFB control was reported and assessing the bio-availability of phage in hives (Ribeiro, Correia, et al., 2019). The same work suggested the protection of phages (e.g.: through encapsulation) to increase their efficacy of action. Other authors had already supported this strategy to other fields (Abdelsattar, Abdelrahman, Dawoud, Connerton, & El-Shibiny, 2019; Malik et al., 2017; Richards & Malik, 2021a, 2021b). The polymer, Eudragit S100, which is pH-responsive, had positive results on phage protection through the different pH ranges in the gastrointestinal tract (Choińska-Pulit, Mituła, Śliwka, Łaba, & Skaradzińska, 2015; Richards & Malik, 2021a, 2021b; Vinner & Malik, 2018; Vinner, Vladisavljević, Clokie, & Malik, 2017). This polymer is able to protect the phage from pH values below 7, when the carboxylic acid groups are protonated and the matrix is tightened. On the other hand, at pH above 7, the deprotonated carboxylic acid groups makes the matrix to be more flexible, allowing the release of the phage.

In this study, differences between administering free or encapsulated (in Eudragit S100) *P. larvae* phage (vB_PlaP_API480) to hives will be analysed. In addition, the efficiency of phages vB_PlaP_API480 (API480) and vB_PlaS_CEB16 (CEB16) to reduce *P. larvae* load inside infected lab-reared larvae will be evaluated.

5.2 Material and methods

5.2.1 *P. larvae* growth conditions and production of spores'

The production of spores was performed as previously described by Alvarado et al, (2013) (Alvarado et al., 2013). For that, the previously isolated *P. larvae* strain PI02-27 (A. Oliveira et al., 2015) was firstly cultivated in MYPGP agar (10 $g.L^{-1}$ Mueller-Hinton Broth (Oxoid); 15 $g.L^{-1}$ yeast extract (Oxoid); 3 $g.L^{-1}$ de K_2HPO_4 (LabKem); 1 $g.L^{-1}$ de Sodium-pyruvate (Fisher); 2% glucose (Ameresco) and 17 $g.L^{-1}$ agar (VWR)) and incubated at 37 °C under 5% CO_2 overnight (O/N), for spore production the plates stayed 10 days in these conditions.

The resulting bacterial cultures were scraped using a spreader and soaked with 2 mL of ice-cold water. Spores were pelleted by centrifugation (5 minutes, 9000 *×g*, 4 °C) and resuspended in fresh water with three washing steps until obtain one final mL. To separate spores from bacterial cells, bacterial content was centrifuged (35 minutes, 7400 *×g*, 4 °C) through the use of a 20%–to–50% Histodenz (Sigma) gradient (Alvarado et al., 2013). The spore fraction (pellet in the bottom) was collected, and five times washed with ice-cold water. The spore quantification was performed through colony forming units (CFU) count, and assessed considering that only 10% of spores naturally germinate (Alvarado et al., 2013; Forsgren et al., 2008).

5.2.2 Bacteriophage production

The propagation of two phages, the previously reported API480 (Ribeiro, Melo, et al., 2019) and the previously isolated CEB16 (unpublished) were performed as described previously (Chapter 3). Briefly, 10 µL of each stored phage suspension were spread evenly using sterile paper strips on lawns of the host strain, PI02-27. Lawns were previously prepared by inoculating the freshly grown strain in 0.4% MYPGP soft-agar. Plates were incubated O/N at 37 °C with 5% CO_2. The soft-agar with confluent phage plaques was then harvested, centrifuged (10 minutes, 9000 *×g*, 4 °C) and filtered-sterilized through 0.22 µm PES membranes. Phage concentration was determined by phage plaque count ($PFU.mL^{-1}$), and phages stocks stored at 4 °C until use.

5.2.3 Phage encapsulation

The API480 was previously encapsulated through freeze drying in Eudragit S100, allowing phage release above pH 7, as previously described (Vinner et al., 2017). The concentration of encapsulated phage was obtained by weighting a sample of 0.5 g of Eudragit S100 capsules and solubilizing it with 1 mL of SM buffer pH 7.5 (5.8 $g.L^{-1}$ NaCl (PanReac); 2 $g.L^{-1}$ $MgSO_4.7H_2O$ (VWR); 50 $mL.L^{-1}$ 1 M Tris-HCl pH

7.5 (VWR)), for 1 hour. Next, after the serial dilutions, the PFU count was determined (PFU.mL^{-1}) and converted to PFU.g^{-1}.

5.2.4 Experimental phage treatments

Two *in vivo* experiments were planned to obtain a preliminary evaluation of the phage performance towards *P. larvae* in hives. Firstly, the possibility of encapsulated API480 to reach larvae (biodistribution) in an infective state after being administered to adult bees (in healthy hives) were evaluated and then, the ability of phages to reduce *P. larvae* load in lab-reared larvae (spore infected).

5.2.4.1 Phage biodistribution assays in hives

The experimental apiary used for this *in vivo* experiment was located in the north of Portugal (Vila Verde). Two different healthy beehives of *Apis mellifera* of similar sizes with brood in the same state of development were used.

Each hive received 300 mL of a different diet in bees' feeders: G1 – 7.6×10^5 PFU.mL^{-1} encapsulated (S100) phage and G2 – 7.6×10^5 PFU.mL^{-1} free phage, both suspended in 50% (w/v) of sucrose solution (pH 5.0). After 24 hours, 20 adult bees and 60 larvae from the first instars (first two-three days after egg hatching) were collected and frozen at -20 °C. RJ was gathered from the bottom of the comb. Both adult bees and larvae were carefully washed 3× with saline solution (0.9% (w/v) NaCl) and dried for 30 minutes before processing. Adult bees were treated as mentioned by Chapter 3, (Ribeiro et al., (2019)) with some adjustments. After removing their wings and legs they were divided into two parts: crops (honey stomach) and mid- hindguts (intestines). The content of each part was carefully removed with the aid of two sterile forceps. Larvae embedded in RJ were weighted (L_RJ). After the first washing, the decanted supernatant (which volume was recorded as first wash) was recovered for phage titration and larvae weight (L) was recorded. The dilution rate (w/v) of RJ (obtained from L_RJ (g) – L (g)) in first wash was assessed for further calculations.

All the samples were well homogenised in SM buffer pH 7.5, with glass beads and vortexing, to promote the phage release (the volume used was recorded for further calculations). After 1 hour a RT, the PFU count was performed as previously. Standard PCR amplifications were used to confirm phage presence. For that, the supernatant of samples after release were directly used, and an 1 µL of internal amplification control (IAC) (targeting *E. coli* LacZ gene) (44 ng.µL^{-1}), was included to avoid false negative results (Ribeiro, Correia, et al., 2019). Both set of primers and amplification conditions, for the IAC and API480 are described in **Table 16**. The mix for PCR had 6.25 µL (1x) Xpert Fast Master mix (Grisp), 1 µL of sample, 1 µL of each primer (5 µM or 10 µM to IAC or CDS_12 API480 respectively) and 3.25 µL

of HyPureTM Molecular Biology Grade water (GE, Healthcare). The PCR products were run in a 1% (w/v) agarose gel and observed under UV light.

Table 16: PCR conditions for IAC confirmation, for API480 presence or host genome integration and for 16S-PCR of *P. larvae* identification.

Target gene	Sequence (5′-3′)	Amplicon size (bp)	PCR conditions
IAC (*LacZ*)	Fw: AGCGAAACCGCCAAGACTGTTA Rev: GTGGATGAAGACCAGCCCTTCC	135	15 minutes 95 °C, 30 seconds 95 °C; 30 seconds 56 °C; 45 seconds, 72 °C (35 cycles) 3 minutes 72 °C
CDS_12 API480	Fw: CAGGAACTCAGACCCTACGC Rev GCCTGCTGCAAAGTCATACA	227	15 minutes 95 °C, 30 seconds 95 °C; 30 seconds 60 °C; 45 seconds 72 °C (35 cycles) 3 minutes 72 °C
16S rRNA	Fw: CTTGTGTTTCTTTCGGGAGACGCC Rev: TCTTAGAGTGCCCACCTCTGCG	1,106	15 minutes 95 °C, 1 minute 95 °C; 30 seconds 55 °C; 1 minute 72 °C (35 cycles) 3 minutes 72 °C

5.2.4.2 Phage efficacy in controlling AFB in lab-reared larvae

The artificial larvae food was prepared as described by Crailsheim et al., (2013), consisting on RJ and a sugar solution of 12% (w/v) D-glucose, 12% (w/v) fructose and 2% (w/v) yeast extract. Just before feeding, the sugar solution was mixed with fresh RJ 1:1 (w/w) and pre-warmed to 35°C. The volume of the diet provided to larvae per day was dependent on the age of the larvae and is described in **Table 17**.

Table 17: Daily volume of food fed to larvae.

	Larvae diet								
Days	0	1	2	3	4	5	6	7	8
Vol. (µl) food per larva	10	10	20	30	40	50	50	60	0

Larvae of the first instars (first two-three days after egg hatching) were grafted from healthy hives, deposited in wells from 48-well plates (VWR) and placed in a previously sterilized desiccator placed in a warm atmosphere (35 °C), 5% CO_2 and relative humidity of 90% (provided by a K_2SO_4 saturated solution and confirmed with a hygrometer). The bottom of the desiccator was filled with 10% (v/v) propolis infusion to avoid the growth of fungal microorganism (Karl Crailsheim et al., 2013; A. Oliveira et al., 2015). For the propolis infusion, 25 g of propolis were suspended in 250 mL sterile water and incubated in the dark for one week at 40 °C with shaking (120 rpm). Then the liquid phase was filtered through a 0.45 µm PES membrane. The experiment took place for 8 days, and the system was opened in the sterile flow chamber.

The arriving day, day 0, was the habituation period for the grafted larvae. The assay started with six groups of 15 to 18 larvae each (**Table 18**). On day 1, spores and phages were mixed with the normal larval diet, according to groups decided based on data from previous reports (Beims et al., 2015; Ghorbani-Nezami et al., 2015; Yost et al., 2016). The groups: C1 B, baseline control group, no spores or phages, used to evaluate the normal rate of mortality; C2 S, the positive control, received only *P. larvae* spores (around 2000 spores of strain ERIC I PI02-27 per larva) to cause AFB disease; C3 P, the phage control, receiving only phages (around 5×10^4 PFU per larva), to assess the phages toxicity for larvae after 9 and 33 hours of API480 and CEB16 respectively; Tt1 received a single dose of phage API480, 9 hours after spore's infection; Tt2 had a repeated dose of phage API480, 9 and 33 hours after infection; Tt3, received two different phages, API480, 9 hours after and CEB16, 33 hours after infection. The application of phages 9 hours after spores infection was based on API480 viability in RJ of 6 hours (Ribeiro, Melo, et al., 2019) and in the fact of spores germinate 12 hours after their ingestion in larval midgut (Yue et al., 2008) allowing phages reach the larvae 3 hours before beginning of spore germination. In the subsequent days the diet did not change (**Table 17**).

Table 18: Control groups and treatments evaluated on lab-reared larvae (spores and phages doses) and the respective number of larvae in each one at day 0 and day 1.

	No. of Larvae		**Spores**	**Phages**	
	Day 0	Day 1	1st dose (0h)	1st dose API480 (9h)	2nd dose phage (33h)
C1 B	15	12	-	-	-
C2 S	18	8	+	-	-
C3 P	10	10	-	+	+ CEB16
Tt1	15	13	+	+	-
Tt2	17	11	+	+	+ API480
Tt3	18	15	+	+	+ CEB16

Larvae were daily monitored, according to their aspect (C-shape length), food consumption, and colour change (A. Oliveira et al., 2015). Larvae with remains of food in the bottom of the well and with no increasing in C-length were marked and considered dead. At the end of the assay, the rate of mortality per group was recorded.

The quantification of *P. larvae* and phages were performed by CFU and PFU counts respectively (Ribeiro, Correia, et al., 2019). Each larva was washed 3× with 0.9% (w/v) NaCl, collected to a tube, homogenized in fresh saline solution and spin centrifuged (the volume used and the weight was recorded

for further calculations). According to the level of infection, each larva was scored as 0 (≤200 CFU/larva) or 1 (>200 CFU/larva). Around five bacterial colonies were propagated five times in fresh medium to remove attached phages. Then, the PCR to amplify phage was performed directly from bacterial colonies. Primers for 16S rRNA (amplicons of 1106 bp) were used to confirm the presence of *P. larvae* strain (Dobbelaere et al., 2001) and primers CDS_12 API480 (amplicons of 227 bp) to verify the integration of API480 on the host genome (Ribeiro, Melo, et al., 2019) (**Table 16**). For the PCR mix, 6.25 µL (1x) Xpert Fast Master mix (Grisp), 1 µL of each primer (10 µM for both 16S rRNA and CDS_12 API480 primers) and 4.25 µL of HyPureTM Molecular Biology Grade water (GE, Healthcare) were used. Result were analysed in a 1% (w/v) agarose gel under UV light.

For phage quantification, each sample of 50 µl homogenized larva was added to 10 µl do chloroform, vortexed, centrifuged (1 minute, 9000 *×g*) and upper phase collected to made serial dilutions in SM buffer (Ribeiro, Correia, et al., 2019). The phage quantification ($PFU.mL^{-1}$) was made as described above.

To detect CEB16 or API480 insensitive mutants inside larvae, one bacterial colony of each larva of the control C2 S and from all treatment groups were propagated five times in solid media as previously mentioned. This test was performed with a drop of 10 µl of each phage (API480 and CEB16 at 2.5×10^7 $PFU.mL^{-1}$). After drying, a line using part of the colony's biomass was done crosswise on phage path and incubated O/N at 37 °C with 5% CO_2. The absence of bacterial growth at the intersections indicated sensitivity to phage (i.e., no phage integration). On the other hand, the presence of grown biomass at the intersection indicated the presence of phage-insensitive mutants (i.e., phage integration in host genome).

5.2.5 Statistical analysis

The statistical analysis of the results was performed using GraphPad Prism 7. In the biodistribution assays in hives, and in lab-reared larvae, the standard deviations were determined with 15% of the variance due to the nature of the *in vivo* assays. The results of the biodistribution of phages in each hive were compared with each other with one-way ANOVA, with Turkey's multiple comparison statistical tests. The bees' sections (crops and mid- hindguts) between different hives were also compared using the previous analysis. In the end larval tissues were compared between different hives with a t-test. In these three analyses, the confidence level was 99.9%, (statistic difference if $p>0.001$). Relative to results of lab-reared larvae, comparation between larval weight was used the t-test with a confidence level of 95%, (statistic difference if $p \leq 0.05$). Comparison between CFU and PFU per larvae, depending on score were

performed with two-way ANOVA, with Turkey's multiple comparison statistical tests (confidence level of 95%, statistic differences if $p \leq 0.05$).

5.3 Results

5.3.1 Biodistribution of encapsulated or free phage API480 in hives

Some problems have arisen in the administration of the formulation with encapsulated API480 in bee feeders. The size of the suspended of Eudragit S100 particles in the prepared sugary solution was not homogeneous, as observed under microscopic field, where visible agglomerates larger than 100 µm were detected (**Figure 26A**). Therefore, the larger agglomerates might not have been consumed by bees (**Figure 26B**), leading to lower phage circulating in G1 comparatively to G2 (about 20% less phage). During the experiment, it was still observed that the encapsulated phage suspension took longer to be consumed compared to the formulation of free phages.

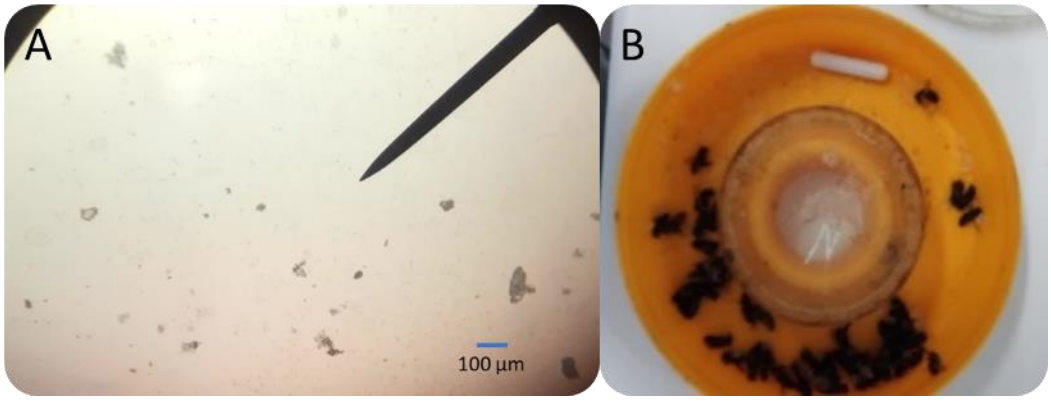

Figure 26: Capsules of API480 after mixing the powder with 50% (w/v) sugar solution. (A) API480 phage capsules were heterogeneous under microscopic observation (scale bar is 100 µm). (B) Solid residues present in the bee's feeder after 24 hours.

After analysis, viable phages were observe in all bee sections (crop, mid- hindgut, and larvae), in both G1 and G2 groups (**Figure 27**). The analysis of the sections from G1 revealed that, crops of adult bees had the higher amount of API480, in average 116 PFU/bee, mid- hindguts had 70 PFU/bee and finally, larvae harboured 5 PFU/larva, all significantly different from each other ($p<0.001$). In G2, the sections with the higher phage amount were the mid- hindguts with 83 PFU/bee, followed by the larvae tissues with 10 PFU/larva and by the crops, with 3 PFU/bee, all also significantly different ($p<0.001$). Regarding RJ, no viable phage was detected in any group. The comparison between the same sections (crops, mid- hindguts, and larvae) from both G1 and G2 groups, showed that only crop section and larvae are significantly different between them ($p<0.001$): crops from G1 with 116 PFU/bee had more phages than the crop of G2 with 3 PFU/bee and also in larvae, G1 had less phages (around 5 PFU/larva), than G2 (10 PFU/larva). Overall, after 24 hours, the bees from G1 had more viable phages (186 PFU/bee) than bees from G2 (86 PFU/bee) ($p<0.001$).

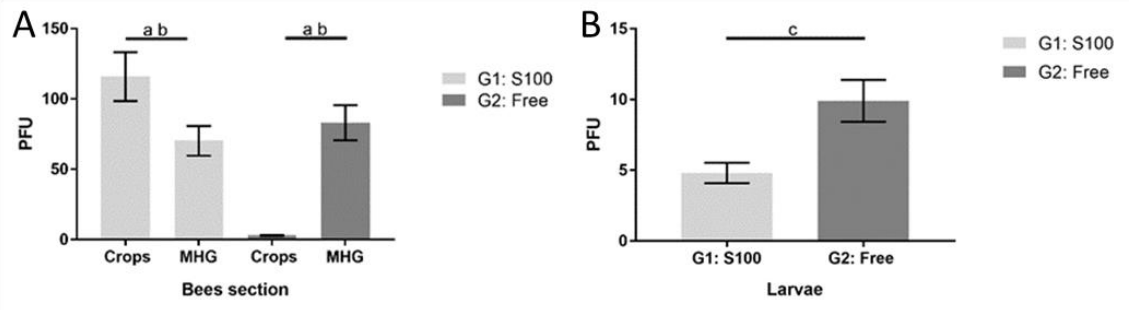

Figure 27: API480 phage biodistribution and bio-availability. The viable API480 phage (PFU) detected in bees' sections crops and mid- hindguts (MHG) (A) and larvae (B) after the 24 hours feed with both diets, the G1 hive with encapsulated phage API480 in polymer S100 and in the G2 hive the free phage API480. Each column represents the mean of 4 replicates with 5 adult bees each and the mean of 3 replicates with 20 different larvae with a variance of 15% represented by error bars. Statistical significance, $p<0.001$; "a" indicates differences between bees' sections in the same group; "b" indicates differences in crops sections between both groups; "c" indicates differences in larval tissue between both groups.

Larval tissues and RJ samples were analysed by standard PCR to search for API480 phage DNA presence (**Figure 28**). In both groups, G1 and G2, the presence of genetic material of the API480 phage in the larvae tissue was confirmed. However, in RJ, phage DNA was only detected in G1.

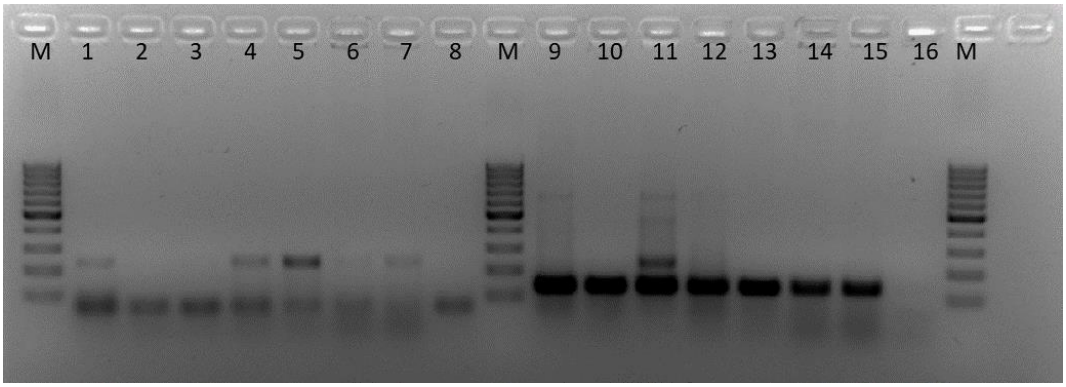

Figure 28: Agarose gel resulting from standard PCR to identify the presence of API480 phage in larvae tissues and in RJ as well as IAC in both diet phage groups G1 (encapsulate S100) and G2 (free phage). Amplification of API480 showed DNA bands of 227 bp, while IAC has DNA bands of 135 bp. M – DNA ladder 100 bp GeneRuler; PCR to API480: 1 – Mix of larvae from G2; 2 – RJ G2; 3 – RJ G2; 4 – Mix of larvae from G1; 5 – RJ G1; 6 – Mix of larvae from G2; 7 – Mix of larvae from G1; 8 – Negative for API480; between the 8 to 16 the order is the same but specific for IAC standard PCR.

5.3.2 Treatment of *P. larvae*-infected lab-reared larvae with phage API480 and CEB16

The *in vivo* effects of API480 and CEB16 were analysed in lab-reared larvae. The corresponding number of larvae in each group in day 1 are in **Table 18** (Supplementary Figure S35).

The percentage of viable and non-viable larvae at the end of the experiment is shown in **Figure 29**. Only groups C1 B and C3 P showed 100% of larvae survival rate. C2 S had only 75%, Tt1 had 85%, Tt3, 80% and finally Tt2 had 55% survival rate.

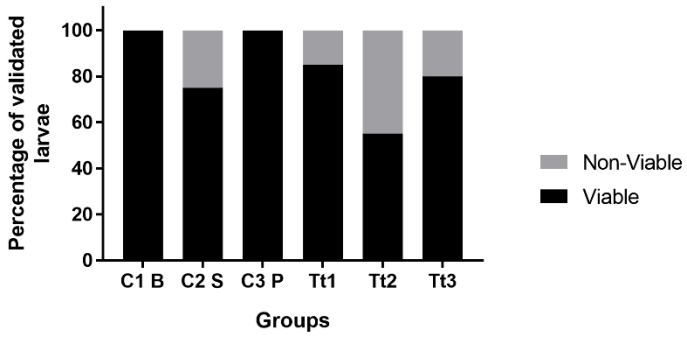

Figure 29: Survival rates of larvae per group on day 8. C1 B: control 1 (no spores and no phages); C2 S: control 2 (only spores); C3 P: control 3 (only phages, API480 and CEB16, 9 and 33 hours after respectively); Tt1: treatment with API480, 9 hours after spore infection; Tt2: treatment with a API480, 9 and 33 hours after spore infection; Tt3: treatment with API480 and CEB16, 9 and 33 hours, respectively, after infection.

The analysis of group C3 P, informed on the absence of phage toxicity to larvae, as no differences were noticed in their weights or length on the last day of the assay (day 8), ($p>0.05$) comparatively to C1 B (**Figure 30**).

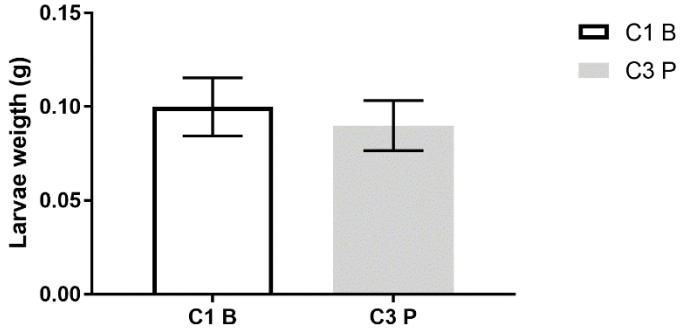

Figure 30: Average larval weight in the 8th day of the assay in group C1 B and C3 P. Each column represents the mean at least 10 different larvae with a variance of 15% represented by error bars. C1 B: control 1 (no spores and no phages); and C3 P: control 3 (only phages, API480 and CEB16, 9 and 33 hours after respectively). Statistical significance, $p<0.05$.

The percentage of larvae from each score, 0 or 1, in the last day of the assay is shown in **Figure 31**. While in controls C1 B and C3 P, all larvae were scored with 0, in C2 S, all larvae were scored with 1 (colonization between 2.3 and 8.3 Log CFU/larva). In the treatment groups, all larvae were also contaminated with *P. larvae*, but the different phage applications led to different bacterial concentrations. In Tt1 group a single application of API480 phage, led to the reduction of the number of infected larvae with score 1, 77% in total, when compared to C2 S ($p<0.05$). Despite still infected, the remaining 23% of larvae were scored as 0. Tt1 larvae had between 1.8 and 8.3 Log CFU/larva. Two sequential applications of API480 in Tt2 had the worse result of the treatments with about 82% of the larvae having score 1, (18% of the larvae, with score 0). Tt2 had between 2.0 and 8.2 Log CFU/larva. In Tt3, where two different phages (API480 and CEB16) were sequentially applied, the results observed evidenced that the highest bacterial load were only in 53% of the larvae, while 47% of them had score 0. In this treatment, the minimum and maximum of load of bacteria detected in a single larva was 1.0 Log CFU/larva and 5.2 Log CFU/larva respectively.

All groups except Tt3 revealed significant differences between larvae with score 0 and score 1 ($p<0.05$) (**Figure 31**). Comparing score 0 from all treatment groups, only Tt3 had significantly different percentage of larvae ($p<0.05$), and comparing score 1 from all groups only C1 B and C3 P, and Tt1 and Tt2 were statistically similar ($p>0.05$) (**Figure 31**).

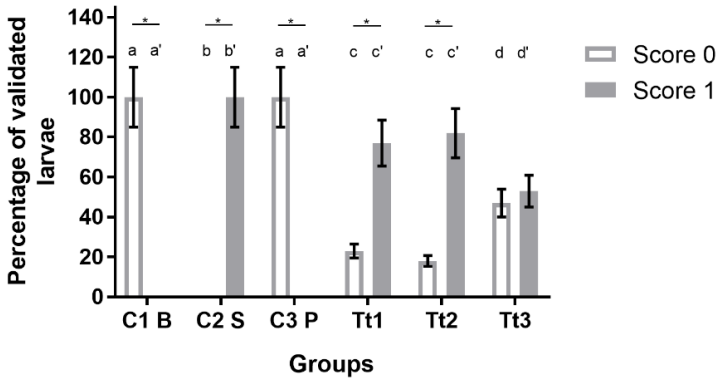

Figure 31: Percentage of larvae scored with 0 or 1 according to the bacterial infection load present in the larvae of controls and treatments after 8 days. The score 0 mean infection lower than 200 CFU/larva or 2.3 Log CFU/larva, while score 1 reflects infection levels upper than 200 CFU/larva or 2.3 Log CFU/larva. C1 B: control 1 (no spores and no phages); C2 S: control 2 (only spores); C3 P: control 3 (only phages, API480 and CEB16, 9 and 33 hours after respectively); Tt1: treatment with API480, 9 hours after spore infection; Tt2: treatment with a API480, 9 and 33 hours after spore infection; Tt3: treatment with API480 and CEB16, 9 and 33 hours, respectively, after infection. Each column represents at least 8 different larvae with a variance of 15% represented by error bars. Statistical significance, $p<0.05$; "*" differences between percentage of larvae with score 0 and 1 in the same group; "a – d" differences on percentage of larvae of score 0 between all groups; "a' – d'" differences on percentage of larvae of score 1 between all groups.

Concerning the average level of bacterial load infection found in larvae of each group (**Figure 32**), In highly infected larvae, C2 S had 5.4 ± 0.8 Log CFU/larva, Tt1 and Tt2 had both 5.8 ± 0.9 Log CFU/larva, respectively and Tt3 had a lower load of *P. larvae* with 3.5 ± 0.5 Log CFU/larva (significantly different $p<0.05$), which is a decrease of 2.0 Log CFU/larva.

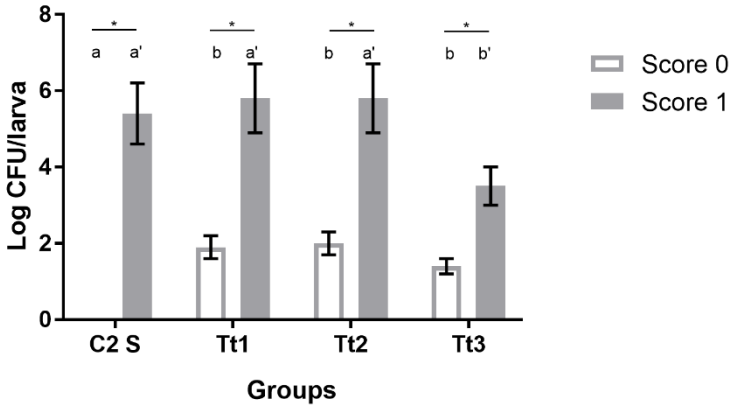

Figure 32: The average load of bacteria detected per larvae with score 0 or 1 after 8 days. C2 S: control 2 (only spores); Tt1: treatment with API480, 9 hours after spore infection; Tt2: treatment with a API480, 9 and 33 hours after spore infection; Tt3: treatment with API480 and CEB16, 9 and 33 hours, respectively, after infection. Each column represents the mean at least 8 different larvae with a variance of 15% represented by error bars. Statistical significance, $p<0.05$; "*" differences between Log CFU/larva with score 0 and 1 in the same group; "a – b" differences on Log CFU/larva of score 0 between all groups; "a' – b'" differences on Log CFU/larva of score 1 between all groups.

Regarding the presence of phages in larvae, no viable phage was detected in C3 P after 8 days (**Figure 33**). However, in all the treatments, the average amount of phages in larvae with score 1, were not statistically different ($p>0.05$), Tt1 had 3.7 ± 0.6 Log PFU/larva, Tt2 had 3.8 ± 0.6 Log PFU/larva and Tt3 had 4.2 ± 0.6 Log PFU/larva. The differences ($p<0.05$) were noted in larvae with 0, between all treatments: Tt1, showed only 0.3 ± 0.1 Log PFU/larva; Tt2, 2.5 ± 0.4 Log PFU/larva and Tt3, 3.5 ± 0.4 Log PFU/larva.

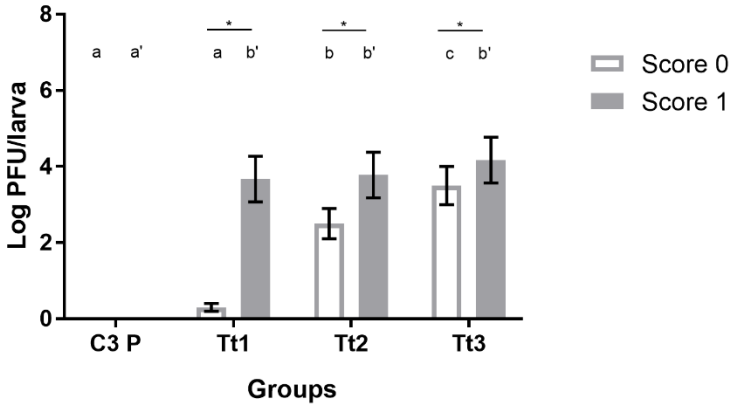

Figure 33: The average quantity of phages per larvae with score 0 or 1 after 8 days. C3 P: control 3 (only phages, API480 and CEB16, 9 and 33 hours after respectively); Tt1: treatment with API480, 9 hours after spore infection; Tt2: treatment with a API480, 9 and 33 hours after spore infection; Tt3: treatment with API480 and CEB16, 9 and 33 hours, respectively, after infection. Each column represents the mean at least 10 different larvae with a variance of 15% represented by error bars. Statistical significance, $p<0.05$; "*" differences between Log PFU/larva with score 0 and 1 in the same group; "a – c" differences on Log CFU/larva of score 0 between all groups; "a' – b'" differences on Log CFU/larva of score 1 between all groups.

After 16S PCR (Supplementary Figure S36), the isolated *P. larvae* colonies were evaluated for the presence of API480 in their genomes (**Figure 34**). In the C2 S no colonies with phage integrated was found, being all sensitive to API480 infection. However, in treatments Tt1 and Tt2, most colonies were insensitive to this phage, having respectively, at least 71% and 73% of the larvae with insensitive colonies. In Tt3, the number of larvae found with resistant colonies decrease to 47%. Conversely, all colonies were sensitive to CEB16 action.

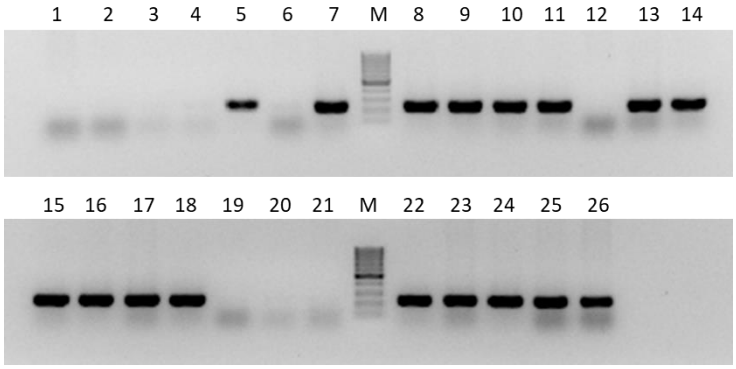

Figure 34: Verification of API480 integration in *P. larvae* colonies cultivated and collected from positive control (C2 S), and all the treatments. Agarose gel showing DNA bands of 227 bp corresponding to API480 only in bacteria derived from the larvae treatments. M – DNA ladder 100 bp GeneRuler; 1 – Mix of *P. larvae* colonies isolated from C1 B larvae; 2 – Mix of *P. larvae* colonies isolated from C2 S larvae; 3 – Mix of *P. larvae* colonies isolated from C2 S larvae; 4 – PI02-27 wild type; 5 – Phage API480; 6 – Mix of *P. larvae* colonies sensitive to API480 isolated from Tt1 larvae; 7 to 11 – *P. larvae* colonies insensitive to API480 isolated from Tt1 larvae; 12 – Mix of *P. larvae* colonies sensitive to API480 isolated from Tt2 larvae; 13 to 18 – *P. larvae* colonies insensitive to API480 isolated from Tt2 larvae; 19 – Mix of *P. larvae* colonies sensitive to API480 isolated from Tt3 larvae; 20 and 21 – *P. larvae* colonies sensitive to API480 isolated from Tt3 larvae; 22 to 26 – *P. larvae* colonies insensitive to API480 isolated from Tt3 larvae.

5.4 Discussion

The use of phages to fight against *P. larvae* is being explored *in vivo* since 2015, either in lab-reared larvae (Beims et al., 2015; Ghorbani-Nezami et al., 2015; Yost et al., 2016) or in hives (Brady et al., 2017). However, a previous study on phage distribution in hives after being administered orally to honeybees, an important form of phage delivery, revealed some drawbacks: T7 phage (used as a model) become non-infective after contacting larvae food, RJ (Ribeiro, Correia, et al., 2019). Furthermore, studies with lab-reared larvae did not explore the appearance of *in vivo* phage host resistant mutants and the impact of administering temperate phages to larvae. Therefore, the aims of the present assays were, to firstly assess if encapsulation brings more viable phages to larvae, and then, to investigate if, when inside larvae phages are effective in controlling *P. larvae*, even being temperate.

To meet the first aim, the biodistribution of phage API480, either free or encapsulated (Eudragit S100) was monitored in healthy hives. The analysis of bee sections (crops and mid- hindguts) from both groups, 24 hours after administration, generally suggested the presence of more phages in adult bees from G1 than from G2 (G1: 186 PFU/bee and G2: 86 PFU/bee). Such differences can be the result of an efficient protection of the viability of phages by polymer Eudragit S100. Particularly, in bee crops, with a lower pH around 4.5–5.5 (Colibar et al., 2010; Zheng et al., 2017) and the presence of anti-viral phenolic compounds or enzymes like proteases (Pasupuleti et al., 2017), encapsulation proved to protect phages. The harmful effect of the crop content in API480 viability was supported by previous *in vitro* assays (Chapter 3), when 1.2 and 3.2 Log reduction $PFU.mL^{-1}$ occurred 6 and 24 hours, respectively, after homogenizing that compartment with the free phage. In mid- hindguts the average phage amount in G1 was similar to G2 ($p>0.001$).

Yet, the opposite result observed in larvae (more phages in G2, 10 PFU/larva than in G1, 5 PFU/larva ($p<0.001$)), were not expected in face of results for bees. In fact, previous results reported losses in free API480 infectivity 6 hours after contact with the RJ (in which phages are directly immersed in brood combs) (Ribeiro, Melo, et al., 2019). Moreover, the phage protection given by S100 was already demonstrated in other studies, for example with *E. coli* phage T3 (Richards & Malik, 2021a, 2021b), *C. difficile* phage CDKM9 (Vinner et al., 2017) or in a *Salmonella enterica* Felix O1 phage (Vinner & Malik, 2018).

The fact that the sugar syrup took longer to be consumed in G1 (for physical reasons bees might have some difficulty in consuming the formulation due to the heterogeneity of particle sizes), might have delayed larval feeding, and can explain such discrepancy. The delay on the consumption might be

supported by Pernal and Currie (2002), that stated that the size of the pollen particles is an important criteria for its collection (bees can select particles smaller than 150 μm, but prefer below 45 μm). Probably, if the experiment was longer (>24 hours), more phages would be recovered in G1.

Summarizing, despite the difficulties, bees from G1 consumed encapsulated API480 phage, and after 24 hours had more phages than bees from G2. Phages reached larvae within 24 hours in both groups, and it can be predicted that if all the diet was consumed and the assay time was extended, more phages would be recovered from G1.

Efforts should be made to improve the formulation of the encapsulated phage, to avoid heterogenicity of the particle size. For example, another method of phage encapsulation, such as nanoemulsions (water-in-oil) using Shirasu Porous Glass (SPG) membranes can be experimented. This lower energy consumption method can allow a better control of the droplet size, due to lower shear forces applied (Joseph & Bunjes, 2014; Kukizaki, 2009).

In the subsequent experiment, aiming to evaluate phage efficacy in reducing *P. larvae* load inside larvae, besides API480, also phage CEB16 was provided to lab-reared larvae. As expected, the evaluation of harmful effects (in C3 P) of phages to larvae confirmed their innocuity. The same absence of negative effects had already been reported (Beims et al., 2015; Brady et al., 2017; Ghorbani-Nezami et al., 2015; Yost et al., 2016).

Results indicated that the administration of a second dose of API480 (Tt2) did not seem to enhance the therapeutic effect comparatively to a single dose (Tt1). Unexpectedly, the percentage of highly contaminated larvae (score 1) were similar ($p>0.05$) between these groups and C2 S. Also, both treatments had high percentage of API480-resistant colonies. One of the reasons that may explain this result, is related with the temperate nature of API480 that integrates into hosts genome (as observed in isolated host colonies), making most of the population of *P. larvae* insensitive to its action (Ribeiro, Melo, et al., 2019), probably through the mechanism of superinfection exclusion (Touchon et al., 2016). Currently, all known *P. larvae* phages are lysogenic bringing some long-term problems in the efficiency of phage action, also due to the induction of phage-resistant bacteria.

When the second dose of phage was replaced to CEB16 (Tt3), infecting API480-resistant strains (Ribeiro, Melo, et al., 2019), promising effects were observed. The number of larvae with score 1 significantly decreased to 53%, comparatively to with the 100% of C2 S, and the bacterial load decreased about 2.0 Log CFU/larva ($p<0.05$). It might, thus, be inferred that the ability of CEB16 to target, not only the wildtype host, but also API480-resistant bacteria was determinant for these results and suggests that

the subsequent administration of phages with different phage receptors (lytic spectra) in hives can be the wisest strategy to control *P. larvae*, especially if temperate phages are used. Despite the use of exclusively virulent phages for therapy is the recommended strategy, if such type of phages are difficult obtain (as happens for *P. larvae*) or if the available temperate phages are difficult to manipulate genetically to remove integration genes (Drulis-Kawa, Majkowska-Skrobek, Maciejewska, Delattre, & Lavigne, 2012; Monteiro et al., 2018), the use of temperate phages can be considered. This has already been described in *in vivo* experiments aiming *P. larvae* control (Brady et al., 2017; Ghorbani-Nezami et al., 2015; Yost et al., 2016), *Clostridium difficile* biofilm formation in *Galleria mellonella* model (J. Y. Nale, Chutia, Carr, Hickenbotham, & Clokie, 2016), or to extended mice the lifespan with *C. difficile* infection (J. Y. Nale, Spencer, et al., 2016).

Overall, results allow us to conclude that the oral delivery of encapsulated phages to adult bees may be advantageous on providing viable phage to larvae but with a different encapsulation strategy using the polymer Eudragit S100. The action of a cocktail of phages with different receptors, or the time lapsed administration of these phages, if they are temperate, may be a good strategy to enhance phage effects in larvae.

It is expected that the decrease in bacterial concentration, inside larvae will allow them to establish their usual defence strategies against tissue invasion by *P. larvae* such as increase the thickness and the complexity of the peritrophic matrix (Garcia-Gonzalez & Genersch, 2013; Yue et al., 2008). Also, by decreasing the concentration of bacteria in brood, the number of spores formed subsequently will also decrease and their spreading will be controlled. Anyway, the selection of virulent *P. larvae* phages are always preferable to temperate ones, genetic manipulation can be used to modify temperate phages in order to improve phage therapy in beekeeping.

References

Abdelsattar, A. S., Abdelrahman, F., Dawoud, A., Connerton, I. F., & El-Shibiny, A. (2019). Encapsulation of E. coli phage ZCEC5 in chitosan–alginate beads as a delivery system in phage therapy. *AMB Express*, *9*(1), 87. https://doi.org/10.1186/s13568-019-0810-9

Abedon, S. T., Kuhl, S. J., Blasdel, B. G., & Kutter, E. M. (2011). Phage treatment of human infections. *Bacteriophage*, *1*(2), 66–85. https://doi.org/10.4161/bact.1.2.15845

Adriaenssens, E. M., Van Vaerenbergh, J., Vandenheuvel, D., Dunon, V., Ceyssens, P.-J., De Proft, M., … Lavigne, R. (2012). T4-Related Bacteriophage LIMEstone Isolates for the Control of Soft Rot on Potato Caused by 'Dickeya solani.' *PLoS ONE*, *7*(3), e33227. https://doi.org/10.1371/journal.pone.0033227

Alvarado, I., Phui, A., Elekonich, M. M., & Abel-Santos, E. (2013). Requirements for in vitro germination of Paenibacillus larvae spores. *Journal of Bacteriology*, *195*(5), 1005–1011. https://doi.org/10.1128/JB.01958-12

Bae, Y. J., Wu, J., Lee, H. J., Jo, E. J., Murugaiyan, S., Chung, E., & Lee, S. W. (2012). Biocontrol potential of a lytic bacteriophage PE204 against bacterial wilt of tomato. *Journal of Microbiology and Biotechnology*, *22*(12), 1613–1620. https://doi.org/10.4014/jmb.1208.08072

Bassi, S., Formato, G., Milito, M., Trevisiol, K., Salogni, C., & Carra, E. (2015). Phenotypic characterization and ERIC–PCR based genotyping of Paenibacillus larvae isolates recovered from American foulbrood outbreaks in honey bees from Italy. *Veterinary Quarterly*, *35*(1), 27–32. https://doi.org/10.1080/01652176.2014.993095

Beims, H., Wittmann, J., Bunk, B., Spröer, C., Rohde, C., Günther, G., … Steinert, M. (2015). Paenibacillus larvae-Directed bacteriophage HB10c2 and its application in American foulbrood-affected honey bee larvae. *Applied and Environmental Microbiology*, *81*(16), 5411–5419. https://doi.org/10.1128/AEM.00804-15

Bigwood, T., Hudson, J. A., Billington, C., Carey-Smith, G. V., & Heinemann, J. A. (2008). Phage inactivation of foodborne pathogens on cooked and raw meat. *Food Microbiology*, *25*(2), 400–406. https://doi.org/10.1016/j.fm.2007.11.003

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of Paenibacillus larvae in honeybee hives. *Journal of Invertebrate Pathology*, *150*(September), 94–100. https://doi.org/10.1016/j.jip.2017.09.010

Carvalho, C., Costa, A. R., Silva, F., & Oliveira, A. (2017). Bacteriophages and their derivatives for the treatment and control of food-producing animal infections. *Critical Reviews in Microbiology*, *43*(5), 583–601. https://doi.org/10.1080/1040841X.2016.1271309

Carvalho, C. M., Gannon, B. W., Halfhide, D. E., Santos, S. B., Hayes, C. M., Roe, J. M., & Azeredo, J. (2010). The in vivo efficacy of two administration routes of a phage cocktail to reduce numbers of Campylobacter coli and Campylobacter jejuni in chickens. *BMC Microbiology*, *10*. https://doi.org/10.1186/1471-2180-10-232

Choińska-Pulit, A., Mituła, P., Śliwka, P., Łaba, W., & Skaradzińska, A. (2015). Bacteriophage encapsulation: Trends and potential applications. *Trends in Food Science and Technology*, *45*(2), 212–221. https://doi.org/10.1016/j.tifs.2015.07.001

Colibar, O., Popovici, D., Eugeniu, C., & Korodi, G. (2010). The effect of acidifiant on the development of bee families (Apis mellifica). *Med. Vet.*, *43*(2), 296–299.

Crailsheim, K., Brodschneider, R., Aupinel, P., Behrens, D., Genersch, E., Vollmann, J., & Riessberger-Gallé, U. (2013). Standard methods for artificial rearing of Apis mellifera larvae . *Journal of Apicultural Research*, *52*(1), 1–16.

https://doi.org/10.3896/ibra.1.52.1.05

Daisley, B. A., Pitek, A. P., Chmiel, J. A., Al, K. F., Chernyshova, A. M., Faragalla, K. M., ... Reid, G. (2020). Novel probiotic approach to counter Paenibacillus larvae infection in honey bees. *The ISME Journal*, *14*(2), 476–491. https://doi.org/10.1038/s41396-019-0541-6

Dobbelaere, W., de Graaf, D. C., & Peeters, J. E. (2001). Development of a fast and reliable diagnostic method for American foulbrood disease (Paenibacillus larvae subsp. larvae) using a 16S rRNA gene based PCR. *Apidologie*, *32*(4), 363–370. https://doi.org/10.1051/apido:2001136

Drulis-Kawa, Z., Majkowska-Skrobek, G., Maciejewska, B., Delattre, A.-S., & Lavigne, R. (2012). Learning from Bacteriophages - Advantages and Limitations of Phage and Phage-Encoded Protein Applications. *Current Protein and Peptide Science*, *13*(8), 699–722. https://doi.org/10.2174/138920312804871193

European Parliament and the Council of the European Union. (2010). Commission regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuff of animal origin. *Official Journal of the European Union*, (L 15/1).

Forsgren, E., Stevanovic, J., & Fries, I. (2008). Variability in germination and in temperature and storage resistance among Paenibacillus larvae genotypes. *Veterinary Microbiology*, *129*(3–4), 342–349. https://doi.org/10.1016/j.vetmic.2007.12.001

Garcia-Gonzalez, E., & Genersch, E. (2013). Honey bee larval peritrophic matrix degradation during infection with Paenibacillus larvae, the aetiological agent of American foulbrood of honey bees, is a key step in pathogenesis. *Environmental Microbiology*, *15*(11), 2894–2901. https://doi.org/10.1111/1462-2920.12167

Genersch, E. (2006). Reclassification of Paenibacillus larvae subsp. pulvifaciens and Paenibacillus larvae subsp. larvae as Paenibacillus larvae without subspecies differentiation. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, *56*(3), 501–511. https://doi.org/10.1099/ijs.0.63928-0

Genersch, E. (2010). American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. *Journal of Invertebrate Pathology*, *103*(SUPPL. 1), S10–S19. https://doi.org/10.1016/j.jip.2009.06.015

Genersch, E. (2017). Beekeeping – From Science to Practice, 157–174. https://doi.org/10.1007/978-3-319-60637-8

Genersch, E., Ashiralieva, A., & Fries, I. (2005). Strain- and Genotype-Specific Differences in Virulence of Paenibacillus larvae subsp. larvae, a Bacterial Pathogen Causing American Foulbrood Disease in Honeybees. *Applied and Environmental Microbiology*, *71*(11), 7551–7555. https://doi.org/10.1128/AEM.71.11.7551-7555.2005

Ghorbani-Nezami, S., LeBlanc, L., Yost, D. G., & Amy, P. S. (2015). Phage Therapy is Effective in Protecting Honeybee Larvae from American Foulbrood Disease. *Journal of Insect Science*, *15*(1), 84. https://doi.org/10.1093/jisesa/iev051

Hawkins, C., Harper, D., Burch, D., Änggård, E., & Soothill, J. (2010). Topical treatment of Pseudomonas aeruginosa otitis of dogs with a bacteriophage mixture: A before/after clinical trial. *Veterinary Microbiology*, *146*(3–4), 309–313. https://doi.org/10.1016/j.vetmic.2010.05.014

Joseph, S., & Bunjes, H. (2014). Evaluation of Shirasu Porous Glass (SPG) membrane emulsification for the preparation of colloidal lipid drug carrier dispersions. *European Journal of Pharmaceutics and Biopharmaceutics*, *87*(1), 178–186. https://doi.org/10.1016/j.ejpb.2013.11.010

Kukizaki, M. (2009). Shirasu porous glass (SPG) membrane emulsification in the absence of shear flow at the membrane surface: Influence of surfactant type and concentration, viscosities of dispersed and continuous phases, and transmembrane pressure. *Journal of Membrane Science*, *327*(1–2), 234–243.

https://doi.org/10.1016/j.memsci.2008.11.026

Kutateladze, M., & Adamia, R. (2010). Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends in Biotechnology*, *28*(12), 591–595. https://doi.org/10.1016/j.tibtech.2010.08.001

Leverentz, B., Conway, W. S., Camp, M. J., Janisiewicz, W. J., Abuladze, T., Yang, M., … Sulakvelidze, A. (2003). Biocontrol of Listeria monocytogenes on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology*, *69*(8), 4519–4526. https://doi.org/10.1128/AEM.69.8.4519-4526.2003

Malik, D. J., Sokolov, I. J., Vinner, G. K., Mancuso, F., Cinquerrui, S., Vladisavljevic, G. T., … Kirpichnikova, A. (2017). Formulation, stabilisation and encapsulation of bacteriophage for phage therapy. *Advances in Colloid and Interface Science*, *249*(March), 100–133. https://doi.org/10.1016/j.cis.2017.05.014

Miyagi, T., Peng, C. Y. S., Chuang, R. Y., Mussen, E. C., Spivak, M. S., & Doi, R. H. (2000). Verification of oxytetracycline-resistant American foulbrood pathogen Paenibacillus larvae in the United States. *Journal of Invertebrate Pathology*, *75*(1), 95–96. https://doi.org/10.1006/jipa.1999.4888

Monteiro, R., Pires, D. P., Costa, A. R., & Azeredo, J. (2018). Phage Therapy: Going Temperate? *Trends in Microbiology*, *xx*, 1–11. https://doi.org/10.1016/j.tim.2018.10.008

Nale, J. Y., Chutia, M., Carr, P., Hickenbotham, P. T., & Clokie, M. R. J. (2016). 'Get in Early'; Biofilm and Wax Moth (Galleria mellonella) Models Reveal New Insights into the Therapeutic Potential of Clostridium difficile Bacteriophages. *Frontiers in Microbiology*, *7*(August), 1–16. https://doi.org/10.3389/fmicb.2016.01383

Nale, J. Y., Spencer, J., Hargreaves, K. R., Buckley, A. M., Trzepin, P., & Douce, G. R. (2016). Bacteriophage Combinations Significantly Reduce Clostridium difficile Growth In Vitro and Proliferation In Vivo. *Antimicrobial Agents and Chemotherapy*, *60*(2), 968–981. https://doi.org/10.1128/AAC.01774-15.Address

Oliveira, A., Leite, M., Kluskens, L. D., Santos, S. B., Melo, L. D. R., & Azeredo, J. (2015). The First Paenibacillus larvae Bacteriophage Endolysin (PlyPl23) with High Potential to Control American Foulbrood. *PLOS ONE*, *10*(7), e0132095. https://doi.org/10.1371/journal.pone.0132095

Oliveira, A., Sereno, R., & Azeredo, J. (2010). In vivo efficiency evaluation of a phage cocktail in controlling severe colibacillosis in confined conditions and experimental poultry houses. *Veterinary Microbiology*, *146*(3–4), 303–308. https://doi.org/10.1016/j.vetmic.2010.05.015

Pasupuleti, V. R., Sammugam, L., Ramesh, N., & Gan, S. H. (2017). Honey, propolis, and royal jelly: A comprehensive review of their biological actions and health benefits. *Oxidative Medicine and Cellular Longevity*, *2017*, 1–21. https://doi.org/10.1155/2017/1259510

Pellegrini, M. C., Alonso-Salces, R. M., Umpierrez, M. L., Rossini, C., & Fuselli, S. R. (2017). Chemical Composition, Antimicrobial Activity, and Mode of Action of Essential Oils against Paenibacillus larvae, Etiological Agent of American Foulbrood on Apis mellifera. *Chemistry and Biodiversity*, *14*(4). https://doi.org/10.1002/cbdv.201600382

Pernal, S. F., & Currie, R. W. (2002). Discrimination and preferences for pollen-based cues by foraging honeybees, Apis mellifera L. *Animal Behaviour*, *63*(2), 369–390. https://doi.org/10.1006/anbe.2001.1904

Ribeiro, H. G., Correia, R., Moreira, T., Vilas Boas, D., Azeredo, J., & Oliveira, A. (2019). Bacteriophage biodistribution and infectivity from honeybee to bee larvae using a T7 phage model. *Scientific Reports*, *9*(1), 620. https://doi.org/10.1038/s41598-018-36432-x

Ribeiro, H. G., Melo, L. D. R., Oliveira, H., Boon, M., Lavigne, R., Noben, J.-P., … Oliveira, A. (2019). Characterization of a new podovirus infecting Paenibacillus larvae. *Scientific Reports*, *9*(1), 20355. https://doi.org/10.1038/s41598-019-56699-

y

Richards, K., & Malik, D. J. (2021a). Bacteriophage Encapsulation in pH-Responsive Core-Shell Capsules as an Animal Feed Additive. *Viruses*, *13*(6), 1131. https://doi.org/10.3390/v13061131

Richards, K., & Malik, D. J. (2021b). Microencapsulation of Bacteriophages Using Membrane Emulsification in Different pH-Triggered Controlled Release Formulations for Oral Administration. *Pharmaceuticals*, *14*(5), 424. https://doi.org/10.3390/ph14050424

Sagona, A. P., Grigonyte, A. M., MacDonald, P. R., & Jaramillo, A. (2016). Genetically modified bacteriophages. *Integr. Biol.*, *8*(4), 465–474. https://doi.org/10.1039/C5IB00267B

Spricigo, D. A., Bardina, C., Cortés, P., & Llagostera, M. (2013). Use of a bacteriophage cocktail to control Salmonella in food and the food industry. *International Journal of Food Microbiology*, *165*(2), 169–174. https://doi.org/10.1016/j.ijfoodmicro.2013.05.009

Sulakvelidze, A., Alavidze, Z., & Morris, J. G. (2001). Bacteriophage Therapy. *Antimicrobial Agents and Chemotherapy*, *45*(31), 649–659. https://doi.org/10.1128/AAC.45.3.649

Summers, W. C., Stent, G., & Twort, F. (2012). The strange history of phage therapy. *Landes Bioscience*, *2*(2), 130–133. https://doi.org/10.4161/bact.20757

Touchon, M., Bernheim, A., & Rocha, E. P. C. (2016). Genetic and life-history traits associated with the distribution of prophages in bacteria. *The ISME Journal*, *10*(11), 2744–2754. https://doi.org/10.1038/ismej.2016.47

Vinner, G. K., & Malik, D. J. (2018). High precision microfluidic microencapsulation of bacteriophages for enteric delivery. *Research in Microbiology*, 1–9. https://doi.org/10.1016/j.resmic.2018.05.011

Vinner, G. K., Vladisavljević, G. T., Clokie, M. R. J., & Malik, D. J. (2017). Microencapsulation of Clostridium difficile specific bacteriophages using microfluidic glass capillary devices for colon delivery using pH triggered release. *PLOS ONE*, *12*(10), e0186239. https://doi.org/10.1371/journal.pone.0186239

Woodrow, A. W. (1942). Susceptibility of honeybee larvae to individual inoculations with spores of *Bacillus* larvae. *Journal of Economic Entomology*, *35*(6), 892–895. https://doi.org/10.1093/jee/35.6.892

Woodrow, A. W., & Holst, E. C. (1942). The mechanism of colony resistance to American foulbrood. *Journal of Economic Entomology*, *35*(3), 327–330. https://doi.org/10.1093/jee/35.3.327

Wu, X. Y., Chin, J., Ghalayini, A., & Hornitzky, M. (2005). Pulsed-field gel electrophoresis typing and oxytetracycline sensitivity of Paenibacillus larvae subsp. larvae isolates of Australian origin and those recovered from honey imported from Argentina. *Journal of Apicultural Research*, *44*(2), 87–92. https://doi.org/10.1080/00218839.2005.11101155

Yost, D. G., Tsourkas, P., & Amy, P. S. (2016). Experimental bacteriophage treatment of honeybees (Apis mellifera) infected with Paenibacillus larvae, the causative agent of American foulbrood disease. *Bacteriophage*, *6*(1), e1122698. https://doi.org/10.1080/21597081.2015.1122698

Yue, D., Nordhoff, M., Wieler, L. H., & Genersch, E. (2008). Fluorescence in situ hybridization (FISH) analysis of the interactions between honeybee larvae and Paenibacillus larvae, the causative agent of American foulbrood of honeybees (Apis mellifera). *Environmental Microbiology*, *10*(6), 1612–1620. https://doi.org/10.1111/j.1462-2920.2008.01579.x

Zheng, H., Powell, J. E., Steele, M. I., Dietrich, C., & Moran, N. A. (2017). Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(18), 4775–4780. https://doi.org/10.1073/pnas.1701819114

Supplementary information

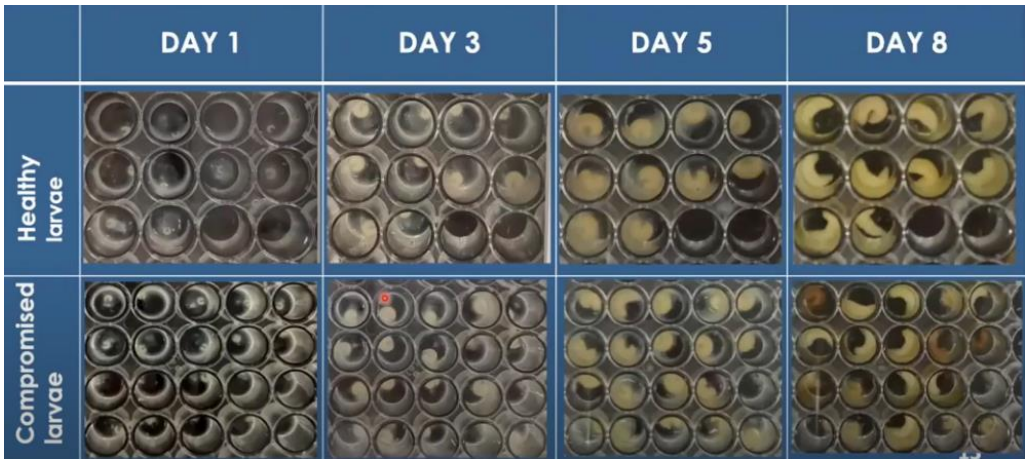

Figure S35: Morphological appearance between the group of healthy larvae and the group with compromised larvae, between day 1 and day 8 of the assay.

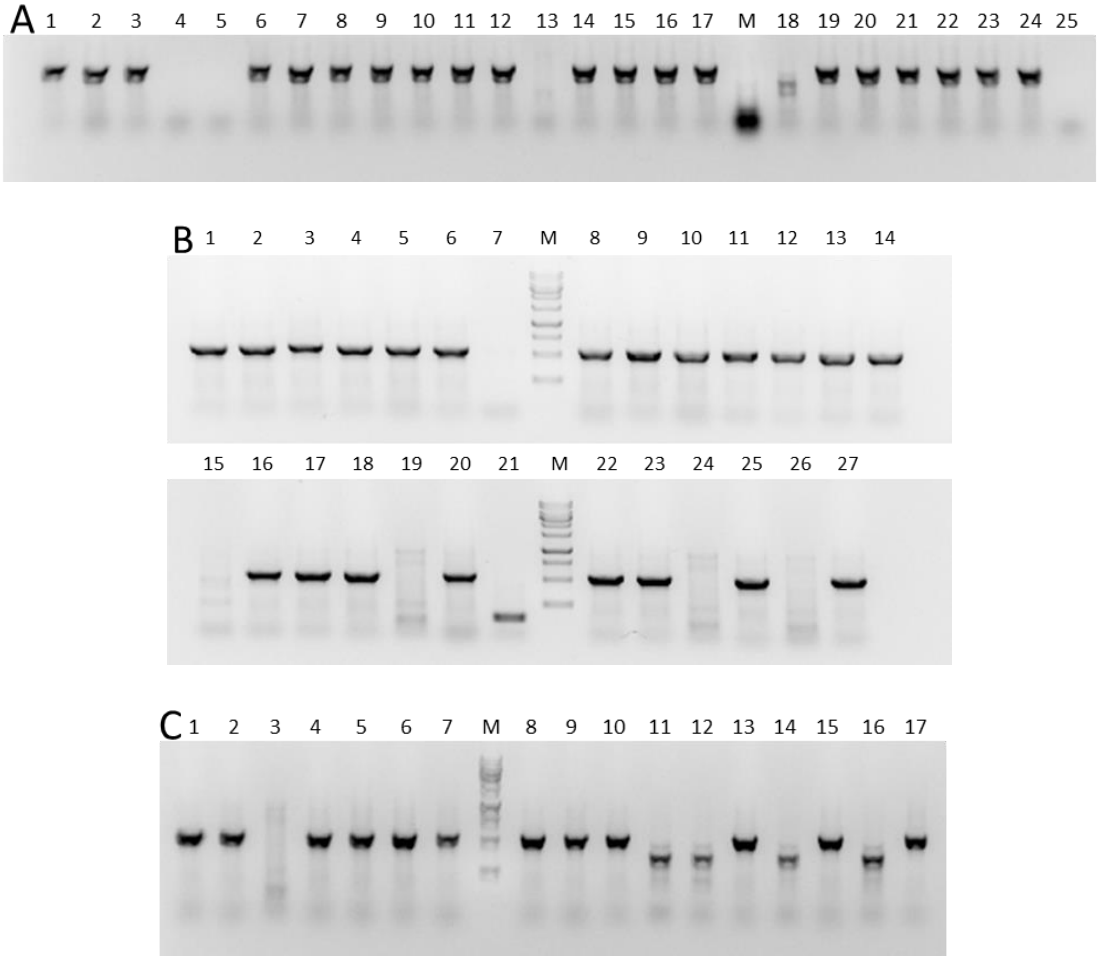

Figure S36: Agarose gels results of 16S standard PCR to identify *P. larvae* strain from (A) C2 S and Tt1, (B) Tt2 and Tt3 and (C) Tt3. The confirmation of *P. larvae* presence on larvae was given by the DNA band of 1,106 bp. M – DNA ladder 1 kb GRS; In A: 1 to 4 – Strains collected from C2 S larvae, 5 and 25 – Negative, 6 to 24 – Strains collected from Tt1 larvae; In B: 1 to 16 – Strains collected from Tt2, 17 to 26 – Strains collected from Tt3, 27 – PI02-27 wild type. In C: 1 to 16 – Strains collected from Tt3, 17 – PI02-27 wild type.

CHAPTER 6: ANALYSIS OF INTACT PROPHAGES IN GENOMES OF *PAENIBACILLUS LARVAE*: AN IMPORTANT PATHOGEN FOR BEES

This chapter was based on the following paper:

Ribeiro HG*, Nilsson A, Melo LDR, Oliveira A. Analysis of intact prophages in genomes of *Paenibacillus larvae*: An important pathogen for bees. *Front Microbiol.* 2022;13 (July). https://doi.org/10.3389/fmicb.2022.903861

Abstract

Paenibacillus larvae is the etiological agent of American Foulbrood (AFB), a highly contagious and worldwide spread bacterial disease that affects honeybee brood. In this study, all complete *P. larvae* genomes available on the NCBI database were analysed in order to detect presence of prophages using the PHASTER software. A total of 55 intact prophages were identified in 11 *P. larvae* genomes (5.0 ± 2.3 per genome) and were further investigated for the presence of genes encoding relevant traits related to *P. larvae*. A closer look at the prophage genomes revealed the presence of several putative genes such as metabolic and antimicrobial resistance genes, toxins or bacteriocins, potentially influencing host performance. Some of the coding DNA sequences (CDS) were present in all ERIC-genotypes, while others were only found in a specific genotype. While CDS encoding toxins and antitoxins such as HicB and MazE were found in prophages of all bacterial genotypes, others, from the same category, were provided by prophages particularly to ERIC I (enhancin-like toxin), ERIC II (antitoxin SocA) and ERIC V strains (subunit of Panton-Valentine leukocidin system (PVL) LukF-PV).

This is the first in-depth analysis of *P. larvae* prophages. It provides better knowledge on their impact in the evolution of virulence and fitness of *P. larvae*, by discovering new features assigned by the viruses.

6.1 Introduction

As the more abundant entities on Earth, bacteriophages (or phages), are considered prime performers in the dynamics of bacterial populations. Phages are generally categorised into two, groups based on their lifecycle: virulent phages (strictly lytic) and temperate phages (lysogenic). The first group have an exclusively lytic lifestyle, always resulting in lysis of the host cell after infection. In the lysogenic cycle, the phage integrates the host genome becoming a prophage and it can remain at this stage for several bacterial generations (L. C. Fortier & Sekulovic, 2013). If external stimuli occur, causing bacterial stress, prophages may be excised from the bacterial chromosome and follow the lytic cycle.

It is widely recognized that temperate phages, capable of interacting with the host genome, are major contributors to the diversity and evolution of most bacterial communities in all ecosystems. The prophage-host interactions are a result of coevolution processes (Harrison & Brockhurst, 2017; Khan, Burmeister, & Wahl, 2020; Koskella & Brockhurst, 2014; Olszak, Latka, Roszniowski, Valvano, & Drulis-Kawa, 2017). On sharing genes, prophages play a key role in modulating bacterial ability to infect their host, to compete with other bacteria and cause disease (virulence), or to adjust metabolism according to environmental conditions in order to survive and grow (fitness). On preventing superinfection events, prophages support the lysogenic state of their hosts and ensure the propagation of their progeny (Bobay, Touchon, & Rocha, 2014; L. Fortier, 2017).

Prophage inputs of new genes into the host can be achieved either by its vertical propagation on bacterial lines or by transduction (horizontal gene transfer, HGT), i.e., when fragments of bacterial DNA are wrongly packed inside phage capsids and then propagated among infected bacteria. For example, prophages can influence traits such as resistance to starvation, biofilm formation, antibiotic tolerance or improved toxicity (Costa, Monteiro, & Azeredo, 2018; L. C. Fortier & Sekulovic, 2013; Touchon, Moura de Sousa, & Rocha, 2017). Several reports have revealed that the presence of prophages can increase the virulence and the toxicity of a bacterial host in many ways. For example, nonvirulent strains of *Escherichia coli*, *Vibrio cholerae*, and *Clostridium botulinum* have become virulent by acquiring prophages with toxin genes (Shiga toxin, Cholera toxin, and Botulinum toxin, respectively) (Barksdale & Arden, 1974; O'Brien et al., 1984; Waldor & Mekalanos, 1996). Further, *Streptococcus mitis* holds adhesion factors encoded by tail genes carried by prophages and *Salmonella enterica* gained enzymes such as superoxide dismutase and neuraminidase, which improves the antioxidant ability and their virulence (Bensing, Siboo, & Sullam, 2001; Feiner et al., 2015; Figueroa-Bossi, Uzzau, Maloriol, & Bossi, 2001). Prophages can protect the lysogenic bacteria against further infections by similar phages and confer an advantage against

competing non-lysogenic bacteria by hampering or delaying their colonization through prophage induction (Brussow, Canchaya, & Hardt, 2004).

Enzymes such as integrases, recombinases or excisionases combine homologous DNA sequences between temperate phage and bacteria genome (Feiner et al., 2015; Lewis, 2001). This mechanism can occur randomly in the host genome, a strategy used for example by the Mu phage, or at specific and conserved locations in the genome, such as for the λ phage (Bondy-Denomy & Davidson, 2014; Toussaint & Rice, 2017).

In nature, the continuous presence of a prophage genome in a bacteria often leads to degradation of genetic sequences, a phenomenon called "phage domestication", also known as Muller's ratchet (Touchon, Bobay, & Rocha, 2014). The host genome seems to inactivate the newly integrated phages and then get rid of undesirable genes by means of genetic degradation (point mutations and deletions) on genetic regions not under selection (Bobay et al., 2014). This mechanism can justify why most prophage sequences usually found in bacterial genomes are incomplete and do not contain essential genes for phage-host interaction (e.g., integrases, endolysins) or lack genes coding for essential structural proteins (Bobay et al., 2014; Casjens, 2003; Czajkowski, 2019). The rates of genetic decay rates seem to be dependent on bacterial robustness, but, for example, in *E. coli*, they have been described as slow (Bobay et al., 2014).

Paenibacillus larvae is a spore-forming Gram-positive bacterium that causes the most severe bacterial honeybee brood disease, American Foulbrood (AFB) (Genersch, 2010). AFB is associated with great economical losses in apiculture, as current legislation does not allow European beekeepers to use antibiotics (European Parliament and the Council of the European Union, 2010) and in many European countries it is mandatory to burn all colonies showing disease signs. The severity of AFB varies with the *P. larvae* genotype involved in the infection (Genersch, Ashiralieva, & Fries, 2005). Five different genotypes, ERIC-types (Enterobacterial Repetitive Intergenic Consensus), have been identified for *P. larvae* so far: ERIC I and II are frequently found in AFB outbreaks; ERIC III and IV have lower epidemiological relevance as they are rarely found; ERIC V is a recently isolated and identified genotype (Beims et al., 2020; Rauch, Ashiralieva, Hedtke, & Genersch, 2009). The pathogenesis of *P. larvae* varies between each genotype and depends on the functional toxins genes and secondary metabolites of the genotypes (Ebeling, Knispel, Hertlein, Fünfhaus, & Genersch, 2016; Genersch, 2017; Müller, Garcia-Gonzalez, Genersch, & Süssmuth, 2015).

Although phages have been proposed to be valuable solutions for mitigation of AFB. All 50 *P. larvae* phages reported to date are temperate (Abraham et al., 2016; Beims et al., 2020, 2015; Carson et al.,

2015; Bryan D Merrill et al., 2018; A. Oliveira, Melo, Kropinski, & Azeredo, 2013; Ribeiro et al., 2019; Tsourkas et al., 2015; Walker et al., 2018; Yost et al., 2018), and, to our knowledge, there are no studies analysing how these phage genomes cause impact on the host phenotype.

In this study, prophage-like sequences found in all complete *P. larvae* genomes available at GenBank (NCBI) were identified and analysed at the genomic level. The prevalence of such sequences in *P. larvae* genomes and the contribution of the prophages for the evolution of *P. larvae* virulence and fitness are herein explored, as far as the five ERIC genotypes are concerned.

6.2 Material and methods

6.2.1 Data collection

All *P. larvae* genomes deposited on GenBank until April 2020 (a total of 14 chromosomes and 20 plasmids) were analysed (minimum genome coverage of 50x) and named from H1 to H14 (**Table 19**). Prophage regions were named from R1 to Rn, placed after the reference of the respective host.

6.2.2 Detection of prophages in *P. larvae* strains

Prophage sequences were obtained until April 2020, for each of the *P. larvae* accession numbers, using PHASTER (PHAge Search Tool Enhanced Release) webserver (http://phaster.ca/) (Arndt et al., 2016; Zhou, Liang, Lynch, Dennis, & Wishart, 2011) (**Table 19**). PHASTER output distinguished intact, questionable and incomplete phage genomes, depending on the number of coding DNA sequences (CDS) of a region attributable to prophages, and on the presence of phage-related genes. Here, questionable and incomplete prophages were both denominated "defective". After the identification by PHASTER, prophages were manually cured for increased accuracy. In cases where important elements for phage infection were missing, such as the N-acetylmuramoyl-l-alanine amidase (an endolysin, the most conserved gene present in *P. larvae* phages (Stamereilers et al., 2018)), other genes with lysis function, structural genes (e.g., major capsid, tail, tail fiber), holins or DNA packaging genes (small and large terminase subunits), these were not considered as intact prophages.

6.2.3 Identification of potential virulence factors encoded by prophages.

BLASTp was used to assess phage coding sequences (CDS) functions, using default parameters and against tailed phages (tax id: 28883), simultaneously, and Conserved Domains-Search Tool (in Pfam database with E-value cut-off of 1×10^{-5} (Finn et al., 2014)). Complete genomes were checked for antibiotic resistance genes through the Resistance Gene Identifier (RGI) of The Comprehensive Antibiotic Resistance Database (CARD), under the "perfect, strict and loose hits" criteria (https://card.mcmaster.ca/analyze/rgi) (Alcock et al., 2020).

To assist with prophage curing and classification, the proteins were grouped into seven functional categories: virion structure, virion assembly, host lysis, DNA replication/ metabolism, gene regulation, host virulence/ fitness functions and lysogeny/ transduction (Stamereilers et al., 2018; Xia & Wolz, 2014).

An adapted Cluster of Orthologous Groups (COG) of proteins were generated by comparing the protein sequences and grouped according to the function. The specific functions of host virulence and

fitness-related proteins were detailed in new categories, as well included the category of unknown functions. It also maintained the six previous categories used in prophages proteins classification.

Special attention was given to CDS with functions that could somehow have influenced host evolution such as those that allow phage lysogeny or transduction and those capable of modulating host virulence or fitness.

6.2.4 Taxonomic classification of prophages

The phage taxonomic family was attributed according to the presence of characteristic structural proteins. For the *Siphoviridae* family (non-contractile tail phages) a set of four proteins close to each other should be present (major tail protein (MTP); two tail assembly proteins (TAP); and tail tape measure protein (TMP)) (Mahony, Alqarni, Stockdale, & Spinelli, 2016; Pell, Kanelis, Donaldson, Lynne Howell, & Davidson, 2009). The presence of the tail sheath protein (TSP) was enough to suggest that prophages belong to the *Myoviridae* family (phages with contractile tail) (Aksyuk et al., 2012; Mahony et al., 2016; Pell et al., 2009; Veesler & Cambillau, 2011), this assumption was also strengthened when three other proteins tail tube protein (TTP); one tail assembly protein (TAP) and tail tape measure protein (TMP), were present close to the TSP and tail fiber proteins. Phages lacking the MTP, TMP, TTP or TSP were classified as *Podoviridae* (phages with non-contractile short tail) (Aksyuk et al., 2012; Veesler & Cambillau, 2011). To support the previous classification, each prophage genome was compared with the GenBank database using BLASTn. In addition, the PHASTER classification presented the phage with the highest number of proteins similar to the one analysed.

6.2.5 Whole genome comparison

The phage genome alignments and the phylogenetic tree were constructed by the MAFFT algorithm (Katoh & Standley, 2013) and Geneious Tree Builder, using the Neighbor-Joining method with bootstrapping of 100 and Tamura-Nei genetic distance model, respectively, present in Geneious R9 (Biomatters, Newark, NJ, USA). All the previously reported *P. larvae* phages (n = 50) (**Table 20**) and all *P. larvae* intact prophages (n = 55) identified here were included in the analysis (**Table 21**). The identity matrix of the phylogenetic trees generated was used to infer on whole identity. Clusters were defined whenever different phages shared at least 60% nucleotide identity and subclusters if the identity was higher than 90% (H. Oliveira et al., 2019; Stamereilers et al., 2018). In case of less than 60% identity with any other phage, it was treated as a singleton.

6.2.6 Statistical analysis

The statistical analysis of the results was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Results were compared using one-way ANOVA, with Turkey's multiples comparison statistical test, in the prevalence and average of each class (total, intact or defective), two-way ANOVA, with Turkey's multiples comparison statistical test, comparisons between both classes of prophages (intact vs defective) and multiple comparisons between the different genotypes for each class of prophages (intact vs defective). For GC content comparison between prophages versus host, the unpaired Welch's t-test was used. All tests were performed with a confidence level of 95%. Differences were considered statistically significant if value of $p \leq 0.05$.

Table 19: *P. larvae* strains and respective reference name, accession number, genome sequencing method and coverage, genotype classification, GC content, genome size, number of contigs and respective sizes. The last three columns refer to the number of total, intact and defective prophages present in each strain, after manual validation. Highlighted in grey, are the hosts with genome size > 4.49 Mbp and with ≥ 7 no. of intact prophages. The second table details data from strains excluded from the analysis.

Host	Strain	Reference strain	Accession No.	Sequencing method	Genome coverage	ERIC genotype	GC%	Size (Mbp)	No. of contigs	Size range of contigs (Kbp)	Prophages (Validated)		
											Total	Intact	Defective
3	DSM 25719	DSM 25719	NZ_ADFW00000000.1	Sanger dideoxy sequencing; 454; Illumina	94	I	44.1	4.58	8	8.1 – 3664	21	8	13
5[a]	MEX14		NZ_LAWY00000000	454	50	I	44.0	4.19	139	0.5 – 213.6	17	3	14
6	ATCC 9545	ATCC 9545	NZ_CP019687.1	PacBio	147.4	I	44.2	4.29	NA	NA	13	5	8
7	ATCC 13537	ATCC 13537	NZ_CP019794.1	PacBio	56.4	IV	44.3	4.41	NA	NA	16	3	13
8	CCM 38	CCUG 7429	NZ_CP020327.1	PacBio	150.8	IV	44.3	4.33	NA	NA	15	5	10
9	SAG 10367	SAG 10367	NZ_CP020557.1	PacBio	214.2	II	44.1	4.67	NA	NA	18	7	11
10	ERIC_I	DSM 7030	NZ_CP019651.1	PacBio; Illumina HiSeq2500	193	I	44.2	4.29	NA	NA	15	5	10
11	ERIC_III	LMG 16252	NZ_CP019655.1	PacBio; Illumina HiSeq2500	114	III	44.2	4.49	NA	NA	18	7	11
12	ERIC_IV	LMG 16247	NZ_CP019659.1	PacBio; Illumina HiSeq2500	113	IV	44.3	4.27	NA	NA	15	3	12
13	DSM 25430; ERIC_II	DSM 25430	NZ_CP019652.1	PacBio; Illumina HiSeq2500	153	II	45.0	4.02	NA	NA	12	1	11
14	ERIC_V	DSM 106052	CP019717.1	PacBio; Illumina HiSeq2500	257	V	44.1	4.67	NA	NA	21	8	13
						Average GC content	44.3			**Total validated**	181	55	126

Host (Excluded)	Strain	Reference strain	Accession No.	Sequencing method	Genome coverage	ERIC genotype	GC%	Size (Mbp)	No. of contigs	Size range of contigs (Kbp)	Prophages (Excluded)		
											Total	Intact	Defective
1[b]	BRL-230010		NZ_AARF00000000.1	454	50	I	44.1	3.98	646	0.25 – 58.6	23	0	23
2[b]	B-3650	LMG 16245	NZ_ADZY00000000.3	Sanger; Illumina	1; 100	I	44.1	4.35	353	0.05 – 331.7	9	0	9
4[c]	DSM 25430	DSM 25430	NC_023134.1	Sanger dideoxy sequencing; 454; Illumina	64	II	45	4.05	NA	NA	8	0	8

[a] Host 5 (MEX14) classified as ERIC I without experimental validation; homology and position in the ERIC I branch of phylogenetic tree available on NCBI database. [b] The high number of contigs available restricted an accurate analysis. [c] Sequence reported by Djukic et al., 2014 (DSM 25430) is identical to the obtained latter (re-sequenced) by Beims et al., 2020. NA: Not applicable.

Table 20: *P. larvae* phages sequenced and available on NCBI. Phages are grouped by the date of the genome announcement and the GenBank Accession Number.

Date	Phage	GenBank Accession No.	Reference
2013	phiIBB_Pl23	KF010834	(Oliveira, Melo, Kropinski, & Azeredo, 2013)
2015	HB10c2	KP202972	(Beims et al., 2015)
	Diva	KP296791	(Carson et al., 2015)
	Lily	KP296792	
	Rani	KP296793	
	Redbud	KP296794	
	Shelly	KP296795	
	Sitara	KP296796	
	Fern	KT361649	(Tsourkas et al., 2015)
	Willow	KT361650	
	Harrison	KT361651	
	Xenia	KT361652	
	Paisley	KT361653	
	Vegas	KT361654	
	Hayley	KT361655	
	Vadim	KT361656	
	Diane	KT361657	
2016	Tripp	KT755656	(Abraham et al., 2016)
2018	BN12	MG727695	(Walker et al., 2018)

	Kiel007	MG727696	
	Dragolir	MG727697	
	PBL1c [1]	MG727698	
	Pagassa	MG727699	
	Tadhana	MG727700	
	Leyra	MG727701	
	Likha	MG727702	
	Wanderer	MH431930	(B.D Merrill et al., 2018)
	Yerffej	MH431931	
	Arcticfreeze	MH431932	
	DevRi	MH431933	
	Gryphonian	MH431934	
	Honeybear	MH431935	
	Kawika	MH431936	
	Lucielle	MH431937	
	C7Cdelta	MH431938	
	Ash	MH454076	
	Bloom	MH454077	
	Eltigre	MH454078	
	Jacopo	MH454079	
	Ley	MH454080	
	LincolnB	MH454081	
	Genki	MH454082	

	Saudage	MH454083	
	Toothless	MH454084	
	Unity	MH460824	(Yost et al., 2018)
	Scottie	MH460825	
	Heath	MH460826	
	Halcyone	MH460827	
2019	vB_PlaP_API480	MK533143.1	(Ribeiro et al., 2019)
2020	phiERICV	CP019719.1	(Beims et al., 2020)
2021	vB_PlaP_SV21 (2)	MZ218124	(Bozdeveci, Akpınar, & Karaoğlu, 2021)

(1) – PBL1c original isolated in 1984 (Dingman, Bakhiet, Field, & Stahly, 1984) but sequenced in 2018 (Walker et al., 2018).

(2) – *P. larvae* phages isolated and reported in 2021 (Bozdeveci et al., 2021), not included in analysis performed here.

Table 21: List of prophages (n = 55), sizes, locations, GC% content, number of proteins and respective percentage of prophages occupation in the host genome.

Prophage	Size prophage (kb)	Genome location	GC %	No. Proteins	No. Phage hit Proteins	No. Hypothetical Proteins	No. Bacterial Proteins	Genes per 1000 bp	Prophage occupation (%)
H3_R2	34,5	681173-715670	44,38	48	45	1	1	1,39	9,71
H3_R3	108,1	831955-940117	44,9	159	140	18	1	1,47	
H3_R5	23,6	1143970-1167596	42,02	42	41	1	0	1,78	
H3_R6	66,1	1356508-1422655	47,76	86	78	8	0	1,30	
H3_R11	62,1	3162110-3224300	42,61	69	62	7	0	1,11	
H3_R14	45,5	3306542-3352032	43,1	79	73	5	1	1,74	
H3_R15	54,4	3581259-3635668	41,35	75	62	12	1	1,38	
H3_R18	50,4	4317267-4367698	42,68	50	48	2	0	0,99	
H5_R2	71,8	939245-1011069	41,94	84	73	11	0	1,17	4,02
H5_R6	40,7	3037678-3078354	43,11	51	45	6	0	1,25	
H5_R13	55,9	3924820-3980782	42,57	79	52	27	0	1,41	
H6_R1	50,4	384329-434773	42,68	50	48	1	1	0,99	6,00
H6_R3	45,6	567210-612826	40,28	71	71	0	0	1,56	
H6_R6	65,4	1390736-1456212	47,09	77	72	5	0	1,18	
H6_R7	50,4	1687454-1737901	44,06	65	57	8	0	1,29	
H6_R8	45,5	1956974-2002463	43,1	75	69	5	1	1,65	
H7_R1	57,3	88-57405	48,33	86	73	12	1	1,50	3,61
H7_R6	40,4	1473939-1514356	40,09	57	54	1	2	1,41	
H7_R10	61,7	3174514-3236266	41,59	58	53	4	1	0,94	
H8_R2	53,7	406153-459928	40,54	65	56	9	0	1,21	5,82
H8_R6	55,7	913539-969255	42,48	64	55	5	4	1,15	

H8_R7	44,1	984737-1028855	43,57	63	60	2	1	1,43	
H8_R8	40,4	1152782-1193204	43,09	56	53	1	2	1,39	
H8_R12	58,1	3606955-3665135	48,26	82	69	13	0	1,41	
H9_R3	48,9	139657-188607	41,67	76	69	7	0	1,55	7,75
H9_R4	69,1	561067-630176	41,16	86	76	10	0	1,24	
H9_R5	60,2	783750-843985	43,02	81	71	9	1	1,35	
H9_R8	34,3	1244130-1278477	47,43	50	40	10	0	1,46	
H9_R10	37,9	2926094-2964064	46,51	54	44	10	0	1,42	
H9_R14	52,2	3581986-3634265	40,98	75	65	10	0	1,44	
H9_R15	59,2	3759316-3818534	41,01	84	75	9	0	1,42	
H10_R4	45,6	1225459-1271112	43,61	73	73	0	0	1,60	5,58
H10_R5	54,4	1500351-1554760	41,35	69	59	9	1	1,27	
H10_R7	55,6	1783038-1838681	48,24	77	72	5	0	1,38	
H10_R10	45,6	2612626-2658322	40,28	70	70	0	0	1,54	
H10_R12	38,2	2801425-2839627	43,44	49	48	1	0	1,28	
H11_R3	48,5	781533-830040	41,26	55	51	4	0	1,13	7,81
H11_R8	58,1	1983129-2041310	48,26	83	70	12	1	1,43	
H11_R10	52,2	2793355-2845586	40,55	65	56	8	1	1,25	
H11_R12	55,5	3251793-3307294	40,55	67	58	8	1	1,21	
H11_R13	51,6	3343780-3395451	43,39	68	58	5	5	1,32	
H11_R14	44,1	3410981-3455101	43,56	63	60	2	1	1,43	
H11_R15	40,4	3579035-3619457	43,08	57	54	1	2	1,41	

H12_R3	52,1	775998-828193	41,25	55	51	3	1	1,06	3,53
H12_R8	58,1	1999595-2057776	48,26	83	70	12	1	1,43	
H12_R13	40,4	3358511-3398933	43,09	57	54	1	2	1,41	
H13_R11	71	2606614-2677682	48,07	98	79	18	1	1,38	1,76
H14_R2	36,7	676583-713337	46,89	52	40	12	0	1,42	8,53
H14_R3	44,8	750873-795687	43,11	65	57	8	0	1,45	
H14_R7	51,9	1374400-1426330	41,32	72	68	4	0	1,39	
H14_R8	42,3	1543921-1586235	41,92	74	66	8	0	1,75	
H14_R9	64,3	1785306-1849567	47,6	88	73	14	1	1,37	
H14_R10	41,7	1909535-1951277	42,63	69	58	11	0	1,65	
H14_R14	57,5	2988754-3046269	48,29	88	74	14	0	1,53	
H14_R15	59,4	3044287-3103635	41,42	84	68	14	2	1,41	
Average Size prophage	51,9	**Average Prophage GC %**	43,5					**Average Prophage occupation %**	5,83
Despad Size prophage	12,7	**Despad Prophage GC %**	2,6					**Despad Prophage occupation %**	2,45

6.3 Results

6.3.1 Prevalence of prophage sequences in *P. larvae* complete genomes

Despite 14 *P. larvae* genomes being available in the GenBank (NCBI), only 11 were analysed (Host 1 and 2 were both excluded due to genome fragmentation and low-confidence results; Host 4 and 13 had the same genomic sequence, and therefore, only one of them was considered (Host 13, the most recently reported)) and in all of them prophage-like elements were identified (**Table 19** and **Figure 37A**). From a total of 181 prophage-related sequences (174 in chromosomes and seven in plasmids), 71 were intact (70 in chromosomes and one in a plasmid) and 110 were defective prophages (104 in chromosomes and six in plasmids) (**Table 19**). However, the manual curing of these sequences only confirmed 55 intact prophages (all in chromosomes) and consequently included 16 more defective prophages (15 in chromosome and one in a plasmid) (**Figure 37B**). All *P. larvae* genomes harboured at least one intact prophage. The average was 5.0 ± 2.3 prophages per genome (**Figure 37C**), varying in size between 23.6 and 108.1 kbp (**Table 21**). The average GC content of prophages and *P. larvae* genomes was 43.5% ± 2.6 (**Table 21**) and 44.3% ± 0.3 (**Table 19**), respectively, and the former occupied 5.83% ± 2.45 of the latter, (variation between 1.76% (Host 13) and 9.71% (Host 3) (**Table 21**)).

Overall, the larger the *P. larvae* genome, the higher the number of intact prophages were observed: Host 3, 9, 11 and 14, the four largest bacterial genomes (>4.49 Mbp), actually included between seven and eight prophages, while Host 5, 6, 7, 8, 10, 12 and 13, smaller, harboured less than five (**Table 19** and **Figure 38**).

Comparatively intact, the defective prophages were always present in higher number (**Table 19**), and at least eight sequences (average of 11.5 ± 1.8) were identified per genome for all the hosts analysed (**Figure 37A** and **Figure 37C**). No differences between the number of intact or defective prophages per ERIC genotype ($p>0.05$) was observed (**Figure 39**).

This work mainly focused on the analysis of intact prophages, considering that these have a more direct impact on the spreading of new traits to their hosts by completing their lytic cycle.

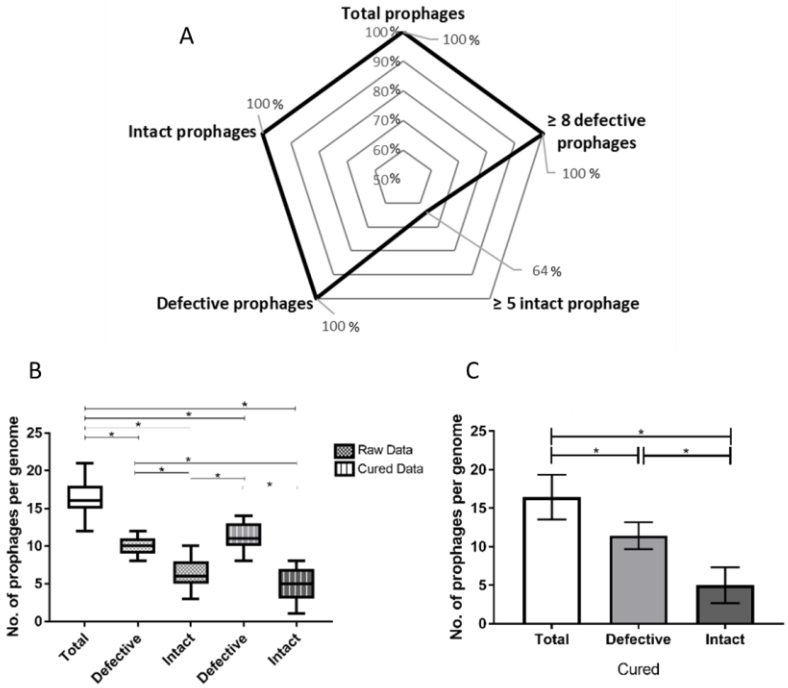

Figure 37: Prophage prevalence in *P. larvae* genomes: (A) Percentage of hosts with ≥ one and ≥ five intact prophages and ≥ one and ≥ eight defective prophages. (B) Whisker plots of prophage frequency per bacterial genome (total, defective and intact) before and after manual curing. Raw data provided directly from PHASTER, cured data results from manual verification. The horizontal line of each box represents the average prophages per genome and the external edges to the minimum/maximum number. (C) Average of total, defective and intact prophages present per host genome. The error bars indicate the standard deviation. Statistically significant, if value of $p<0.05$ (*).

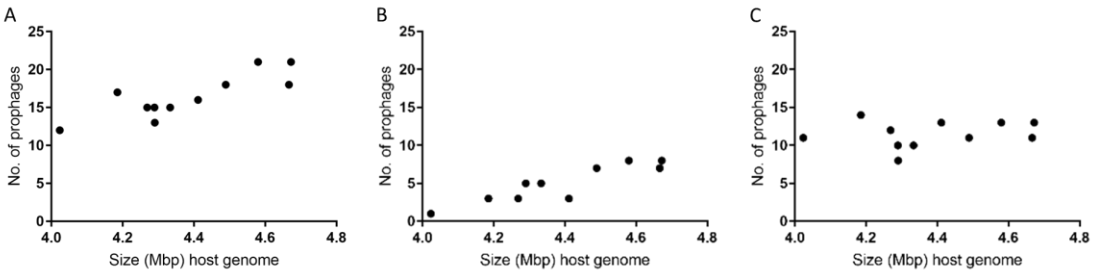

Figure 38: Number of prophages per size of host *P. larvae* genomes: (A) Total (B) Intact (C) Defective.

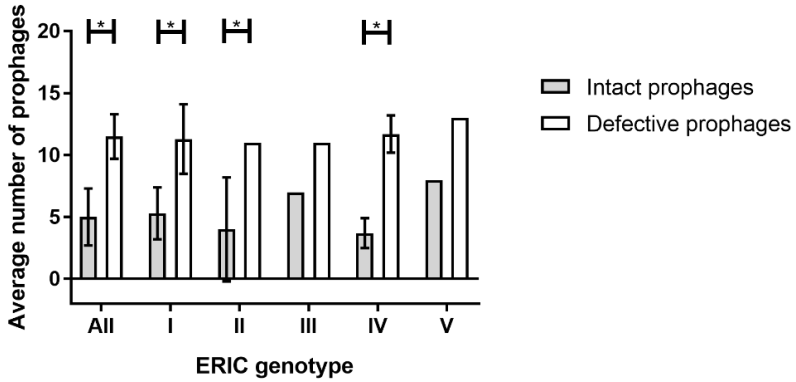

Figure 39: Average number of prophages (intact and defective) present per ERIC genotype (ERIC I-V). The error bars indicate the standard deviation. Statistically significant, if value of $p<0.05$ (*).

6.3.2 Prophage protein library

A total of 3876 CDS were identified among the 55 intact prophage genomes. All CDS were grouped into 36 functional categories using COG. Around 43% of the groups encoded proteins with unknown function. The frequency of CDS per COG is illustrated in **Figure 40**.

The role of the prophage genes was not experimentally confirmed, and therefore, the analysis was conducted relying on the homologies provided by their amino acid (aa) sequences, using BLASTp. In average, each protein had 194 ± 162 aa. The largest, with 1234 aa, was identified as the tail tape measure protein (TMP) and was present in H3_R14, H10_R4 and H10_R10, and the shortest, 28 aa, a HP present in H6_R8, H10_R4, H11_R15 and H12_R13.

About 95% of *P. larvae* prophage CDS have at least one homologous sequence with tailed phages (tax id: 28883) (based on NCBI non-redundant database). Associated with prophage ability to transport and exchange genomic DNA fragments between hosts, transposases seemed to be the most frequently present enzymes in prophages (found 112 CDS encoding them), followed by 39 integrases and 20 recombinases. Together with some regulators, these three enzymes represented 5.7% in the COG analysis (**Figure 40**). A set of 68 phage CDS, globally related to antimicrobial resistance, toxicity for bacteria/larvae (toxin-antitoxin (TA) systems, toxins) or transport of substances, metabolism and germination/sporulation events were subsequently identified as having potential influence on host performance (**Table 22**). Due to the high diversity of host functions associated with virulence and fitness, the percentage of each individual trait was low less than 2% in the COG analysis. TA systems was the category with the highest percentage (1.6%) (**Figure 40**).

Although RGI analysis did not indicate any functional antimicrobial resistance (AMR) gene, AMR-related sequences, such as TetR family transcriptional regulator of a tetracycline resistance mechanism, the β-lactamase superfamily domain (MBL fold metallo-hydrolase YycI) that hydrolyses the β-lactam antibiotics class B or the β-lactamase inhibitory proteins (BLIP), able to inhibit a variety of class A β-lactamases such as the penicillin antibiotics were identified. Few CDS seemed to be also involved in the transport of antibiotics out of bacterial cells (e.g., multidrug efflux small multidrug resistance (SMR) transporter), a mechanism associated with antimicrobial resistance.

There were CDS for other types of transporter proteins, either generic, such as ATP-binding cassette (ABC transporter), the major facilitator superfamily (MFS), efflux, small multidrug resistance (SMR) or very specialized ones- aromatic acid exporter and iron–sulfur (Fe-S) cluster assembly proteins SufB and NifU.

The analysed prophages also harboured TA systems. For example, for the *hicAB* system, consisting of the HicA toxin and HicB antitoxin, both parts were identified, while for *mazEF* or *socAB* systems, only the antitoxin part of the TA cassette was present.

Prophages further possess CDS that putatively confer virulence traits against bee larvae. These include metallopeptidases like enhancin, *Yersinia* outer proteins (Yops) like YopX, a N-acetylglucosamine (GlcNAc)-chitin binding protein (GbpA), the precursor of a subunit of Panton-Valentine leukocidin system (PVL) LukF-PV, a pore -forming epsilon-toxin type B (EtxB), a bacteriocin-like closticin and the DNA internalization competence protein ComEC/Rec2.

The research found CDS for enzymes that may interfere with host metabolism and regulation, such as phosphomannomutases, transglycosylases, a pyruvate dehydrogenase E1 and the histidine kinase-like protein. Finally, our analysis suggested the presence of CDS that can be involved in sporulation and germination, like the outer spore coat protein CotE, sporulation protein YhbH, and spore protease YyaC.

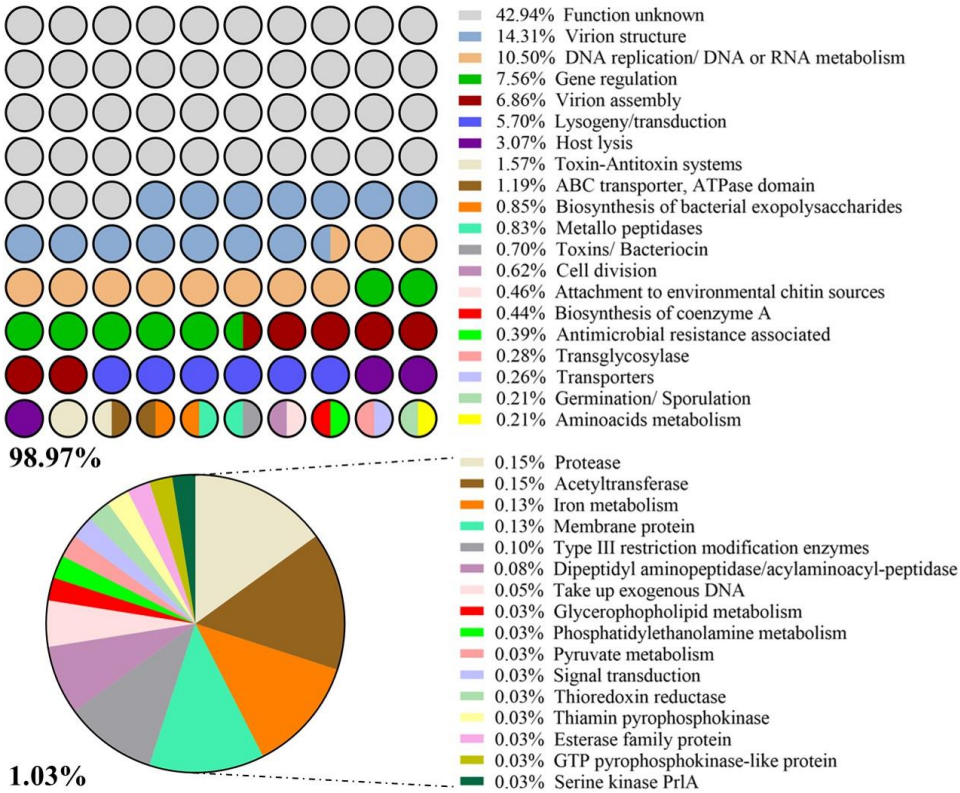

Figure 40: List of categories Cluster of Orthologous Groups (COG). Frequency (%) of prophage-derived CDS with a given function per COG.

6.3.3 Distribution of proteins related to putative host traits according to ERIC genotype

Despite the low number of available genomes representing each of the *P. larvae* ERIC genotypes (**Table 19**), prophages with proteins involved in bacterial fitness (metabolic functions, transport of nutrients, sporulation and germination) or virulence (like toxins, bacteriocins and AMR-related proteins) were identified in all ERIC genotypes, and some were exclusive to a given genotype (**Table 22**). For example, in ERIC I strains, 21 unique proteins were observed, including transporters (an efflux transporter and the DNA internalization protein ComEC/Rec2), a bacteriocin (closticin and enhancin-like protein), enzymes (histidine kinase and pyruvate dehydrogenase E1) and sporulation or germination-related proteins, while in ERIC II strains, the antitoxin SocA, the FtsX-like permease, the MazG-like nucleotide pyrophosphohydrolase, the structural protein involved in spore formation, CotE, and the DNA mismatch repair protein MutS were exclusively present. The proteins identified only in ERIC III strains were YncE, related with DNA binding, and PgpA, a phosphatidylglycerophosphatase, in ERIC IV were involved in iron–sulfur (Fe-S) uptake (SufB) and nitrogen fixation (NifU) and in ERIC V were proteins from the aromatic acid exporter family, a leukocidin subunit LukF-PV precursor, two other toxins, a membrane protein and the chitin-binding protein GbpA.

However, some prophage-derived proteins were found to be shared between genotypes. For example, the pore-forming toxin EtxB and the host-nuclease inhibitor protein Gam, were identified in ERIC I and V strains. ERIC I, III and IV strains share a protein participating in chromosomal partition during cell division (segregation and condensation protein B, ScpB), and other responsible for the racemisation of phenylalanine (phenylalanine racemase). Virulence-associated protein E was only identified in ERIC I and II strains, while ERIC I, II and V share a S8 family serine peptidase and an acetyltransferase. The β-lactamase inhibitory protein (BLIP) and a coat protein were present in both ERIC III and IV strains. From the transporter proteins previously enumerated, the MFS transporter was only present in ERIC I and III strains, while the multidrug efflux SMR transporter was present in ERIC I, III and IV strains. Proteins such as phosphomannomutase, HicB and MazE antitoxins or YopX family protein were identified in all ERIC genotypes, while the transcriptional regulator of the TetR family, the HicA toxin, the transglycosylase and the ImmA/IrrE were present in all except the recently reported ERIC V.

Table 22: CDS identified in prophages potentially influencing host virulence or fitness. The grey colour identifies the corresponding CDS in the respective ERIC genotype. *CDS only found in this genotype.

	ERIC I				ERIC II		ERIC III	ERIC IV			ERIC V
CDS	Host 3	Host 5	Host 6	Host 10	Host 9	Host 13	Host 11	Host 7	Host 8	Host 12	Host 14
TetR family transcriptional regulator (TetR/AcrR)											
β-lactamase superfamily domain (metal β-lactamases (MBL) fold metallo-hydrolase YycI)											
β-lactamase inhibitory proteins (BLIP)											
ATP-binding cassette (ABC) transporter group											
Aromatic acid exporter family protein											*
Major facilitator superfamily (MFS) transporter											
Multidrug efflux small multidrug resistance (SMR) transporter											
Efflux transporter-like protein		*									
Iron–sulfur (Fe-S) cluster assembly protein SufB								*		*	
NifU family iron–sulfur (Fe-S) cluster assembly protein - SUF system								*		*	
metal β-lactamases (MBL) fold metallo-hydrolase											
Phosphomannomutase											
Transglycosylase											
Toxin HicA											
Antitoxin HicB											
Antitoxin MazE											
Antitoxin SocA					*						
AbrB/MazE/SpoVT family DNA-binding domain-containing protein											
Bacterial toxin 44											*
Toxin											
Toxin-like protein											*
Bacteriocin biosynthesis protein		*									

Epsilon-toxin type B (EtxB)											
Leukocidin LukF-PV precursor = leukotoxin											
Closticin	*										
DNA internalization-related competence protein ComEC/Rec2		*	*								
Segregation and condensation protein B (ScpB)											
YopX family protein											
Enhancin / phosphohydrolase		*									
Virulence-associated protein E											
ImmA/IrrE family metallo-endopeptidase											
Esterase family protein		*									
S8 family serine peptidase											
Histidine kinase-like protein		*									
Pyruvate dehydrogenase E1 component subunit alpha		*									
Metallopeptidase											
FtsX-like permease family protein					*						
GlcNAc-chitin binding protein (GbpA)											*
Dipeptidyl aminopeptidase/acylaminoacyl-peptidase											
GTP pyrophosphokinase-like protein	*										
Host-nuclease inhibitor protein Gam (Gam)											
MazG-like nucleotide pyrophosphohydrolase					*						
VRR-NUC domain-containing protein											
YifB family Mg chelatase-like AAA ATPase		*	*	*							
YncE family protein							*				
YxeA family protein											
Coat protein											
Iron-containing alcohol dehydrogenase		*									
Ketopantoate hydroxymethyltransferase											
Membrane protein											*

Outer spore coat protein (CotE)						*					
Spore protease YyaC	*	*	*	*							
Sporulation protein YhbH	*										
Stress protein	*	*	*	*							
DNA mismatch repair protein MutS						*					
ERF superfamily protein											
Acetyltransferase											
dCMP deaminase family protein		*	*								
Murein transglycosylase-like protein		*									
NTP-binding protein	*										
Peptidase domain				*							
Phenylalanine racemase											
Phosphatidylglycerophosphatase A (PgpA)							*				
Phosphatidylserine decarboxylase		*									
STAS-like domain-containing protein		*									
Thiamin pyrophosphokinase		*									
Thioredoxin reductase											*
YqaE/Pmp3 family membrane protein											
	Host 3	Host 5	Host 6	Host 10	Host 9	Host 13	Host 11	Host 7	Host 8	Host 12	Host 14
	ERIC I				**ERIC II**		**ERIC III**	**ERIC IV**			**ERIC V**

6.3.4 Prophage taxonomy

All new phage genomes analysed encode the TMP, and therefore no podoviruses were identified. **Table 23** details the structural proteins in the base of prophage morphology assumptions and subsequent taxonomic classification. Based on the defined criteria, 34 of the 55 prophages were assigned as siphoviruses. Of these 34, four genomes contain all genes encoding structural proteins that distinguish this taxonomic group, 13 did not have one of the proteins (TAP or MTP/TTP) and 17 were described as *Siphoviridae* members. The latter, despite not having both TAP and MTP/TTP also miss the exclusive protein TSP of the *Myoviridae* family. This classification, supported by BLASTn and PHASTER analysis, revealed high homology (E-value = 0.0; Coverage between 29% and 94%, Identity >88.03%) with other previously reported *P. larvae Siphoviridae* phages (Stamereilers et al., 2018).

The remaining 21 prophage genomes, when containing genes encoding the TSP were considered to belong to the *Myoviridae* family. Genome comparison revealed that, while 15 prophages were genetically close to members from the same family, (*Brevibacillus laterosporus* phages Jimmer1, Jimmer2 and Abouo (Sheflo et al., 2013)), the remaining six shared identity (84% – 100%, with a coverage between 12% and 94%) with previously described siphoviruses, such as phage Lily.

Table 23: Taxonomic classification of prophages based on structural proteins present (Y: protein present).

Prophage	TSP	MTP/TTP	TAP	TAP	TMP	Family	Most common phage (PHASTER)	BLASTn parameters			
								Homolog phage	Coverage %	Identity %	E-value
H3_R2					Y	*Siphoviridae*[a]	Vegas (*P. larvae*)	Dragolir	67	99,92	0
H3_R3		Y[#]	Y	Y	Y	*Siphoviridae*	Tripp (*P. larvae*)	Heath	38	90,61	0
H3_R5		Y[#]	Y	Y	Y	*Siphoviridae*	Fern (*P. larvae*)	Jacopo	87	99,15	0
H3_R6			Y	Y	Y	***Siphoviridae***	Tripp (*P. larvae*)	Tripp	75	99,45	0
H3_R11	Y	Y[#]	Y		Y	*Myoviridae*	Lily (*P. larvae*)	Lily	41	84,28	0
H3_R14		Y[#]	Y		Y	***Siphoviridae***	Vegas (*P. larvae*)	Hayley	72	99,98	0
H3_R15	Y	Y*	Y	HP	Y	*Myoviridae**	Jimmer1 (*B. laterosporus*)	Yerffej	11	99,37	0
H3_R18		Y[#]			Y	*Siphoviridae*[a]	Vegas (*P. larvae*)	Vegas	29	99,47	0
H5_R2	Y	Y*	Y	HP	Y	*Myoviridae*	Harrison (*P. larvae*)	Harrison	25	99,97	0
H5_R6		Y[#]			Y	*Siphoviridae*[a]	Vegas (*P. larvae*)	Hayley	32	99,47	0
H5_R13		Y[#]	Y	Y	Y	*Siphoviridae*	Fern (*P. larvae*)	Leyra	38	97,91	0
H6_R1		Y[#]			Y	*Siphoviridae*[a]	Vegas (*P. larvae*)	Vegas	29	99,47	0
H6_R3		Y[#]	Y		Y	***Siphoviridae***	Harrison (*P. larvae*)	Paisley	96	99,98	0
H6_R6			Y	Y	Y	***Siphoviridae***	Tripp (*P. larvae*)	Heath	61	90,57	0
H6_R7	Y	Y*	Y		Y	*Myoviridae**	Jimmer1 (*B. laterosporus*)	Harrison	9	96,25	0
H6_R8		Y[#]	Y		Y	***Siphoviridae***	Vegas (*P. larvae*)	Hayley	72	99,98	0
H7_R1			Y		Y	*Siphoviridae*[a]	Tripp (*P. larvae*)	Scottie	94	99,91	0
H7_R6		Y[#]			Y	*Siphoviridae*[a]	Vegas (*P. larvae*)	Dragolir	36	96,08	0
H7_R10		Y[#]	Y	Y	Y	*Siphoviridae*	Rani (*P. larvae*)	Diva	18	84,37	0
H8_R2	Y	Y*	Y		Y	*Myoviridae**	Jimmer1 (*B. laterosporus*)	Harrison	11	94,73	0
H8_R6	Y	Y*	Y	HP	Y	*Myoviridae**	Abouo (*B. laterosporus*)	Dragolir	22	93,18	0
H8_R7	Y	Y[#]	Y		Y	*Myoviridae*	Lily (*P. larvae*)	Lily	79	91,09	0
H8_R8		Y[#]			Y	*Siphoviridae*[a]	Vegas (*P. larvae*)	Dragolir	36	96,09	0
H8_R12			Y		Y	*Siphoviridae*[a]	Tripp (*P. larvae*)	Scottie	94	99,97	0
H9_R3	Y	Y*	Y		Y	*Myoviridae**	Jimmer1 (*B. laterosporus*)	Wanderer	11	89,43	0

H9_R4	Y	Y*	Y		Y	*Myoviridae**	Jimmer1 (*B. laterosporus*)	Paisley	9	86,88	0
H9_R5		Y#	Y		Y	***Siphoviridae***	Vegas (*P. larvae*)	Diane	41	92,1	0
H9_R8			Y	Y	Y	***Siphoviridae***	Tripp (*P. larvae*)	C7Cdelta	66	91,32	0
H9_R10			Y	Y	Y	***Siphoviridae***	Tripp (*P. larvae*)	Ley	55	89,51	0
H9_R14	Y	Y*	Y		Y	*Myoviridae**	Jimmer1 (*B. laterosporus*)	Sitara	15	86,09	0
H9_R15	Y	Y*	Y		Y	*Myoviridae**	Jimmer1 (*B. laterosporus*)	Leyra	7	97,86	0
H10_R4		Y#	Y		Y	***Siphoviridae***	Vegas (*P. larvae*)	Vadim	99	99,95	0
H10_R5	Y	Y*	Y		Y	*Myoviridae**	Jimmer1 (*B. laterosporus*)	Yerffej	11	99,37	0
H10_R7			Y	Y	Y	***Siphoviridae***	Tripp (*P. larvae*)	Heath	74	90,61	0
H10_R10		Y#	Y		Y	***Siphoviridae***	Harrison (*P. larvae*)	Paisley	96	99,99	0
H10_R12		Y#			Y	*Siphoviridae*@	Vegas (*P. larvae*)	Hayley	34	99,46	0
H11_R3		Y#	Y		Y	***Siphoviridae***	Fern (*P. larvae*)	Likha	18	85,1	0
H11_R8			Y		Y	*Siphoviridae*@	Tripp (*P. larvae*)	Scottie	94	99,98	0
H11_R10	Y	Y*	Y		Y	*Myoviridae**	Jimmer1 (*B. laterosporus*)	Harrison	9	94,91	0
H11_R12	Y	Y*	Y		Y	*Myoviridae**	Abouo (*B. laterosporus*)	Harrison	11	94,73	0
H11_R13	Y	Y*	Y	HP	Y	*Myoviridae**	Abouo (*B. laterosporus*)	Dragolir	23	93,18	0
H11_R14	Y	Y#	Y		Y	*Myoviridae*	Lily (*P. larvae*)	Lily	79	91,09	0
H11_R15		Y#			Y	*Siphoviridae*@	Vegas (*P. larvae*)	Dragolir	36	96,08	0
H12_R3		Y#	Y		Y	***Siphoviridae***	Fern (*P. larvae*)	Yerffej	19	84,49	0
H12_R8			Y		Y	*Siphoviridae*@	Tripp (*P. larvae*)	Scottie	94	99,98	0
H12_R13		Y#			Y	*Siphoviridae*@	Vegas (*P. larvae*)	Dragolir	36	96,09	0
H13_R11			Y		Y	*Siphoviridae*@	Tripp (*P. larvae*)	Scottie	60	88,03	0
H14_R2			Y		Y	*Siphoviridae*@	Tripp (*P. larvae*)	Tripp	52	89,95	0
H14_R3	Y	Y#	Y		Y	*Myoviridae*	Lily (*P. larvae*)	phiERICV	94	100	0
H14_R7	Y	Y*	Y	HP	Y	*Myoviridae**	Jimmer2 (*B. laterosporus*)	Sitara	11	87,86	0
H14_R8	Y	Y*	Y	HP	Y	*Myoviridae**	Jimmer2 (*B. laterosporus*)	Paisley	17	86,26	0
H14_R9			Y		Y	*Siphoviridae*@	Tripp (*P. larvae*)	Heath	67	89,07	0
H14_R10	Y	Y*	Y	HP	Y	*Myoviridae**	Jimmer1 (*B. laterosporus*)	phiERICV	7	94,99	0
H14_R14			Y		Y	*Siphoviridae*@	Tripp (*P. larvae*)	Heath	76	89,11	0

H14_R15	Y	Y*	Y	HP	Y	*Myoviridae*	Harrison (*P. larvae*)	phiERICV	12	95,37	0

TSP: Tail sheath protein; MTP: Major tail tube protein; TTP: Tail tube protein; TAP: Tail assembly protein; TMP: Tail tape measure protein; Y[#]: one of both proteins present; Y*: only TTP present; HP: Hypothetical protein; *Siphoviridae* [a] – prophages from this family based on phage homology of BLASTn; *Siphoviridae* – prophages with the four typical proteins; ***Siphoviridae*** – three of the four typical proteins. *Myoviridae** – prophages homologous to *Brevibacillus laterosporus* phages; *Myoviridae* – prophages homologous to other siphovirus phages.

6.3.5 Whole genome comparison of intact prophages

A previous analysis, grouped *P. larvae* phages into four clusters (Fern, Halcyone, Harrison and Vegas) and two singletons (Lily and API480) (Ribeiro et al., 2019). The re-alignment included these newly identified intact prophages revealed 12 singletons (here S1 to S12) and 22 clusters (here C1 to C22), the latter divided into 51 subclusters (from A to AY) (**Figure 41** and **Table 24**).

This comparison changes the identity between phage genomes adjusts two of the previously reported groups (Ribeiro et al., 2019). The introduction of prophages H3_R4 and H10_R4 resulted in a division of the Vegas cluster into two new clusters, C11 (including H3_R4 and H10_R4) and C12. Besides, comparatively to Harrison, C10 has one more phage, H6_R3. The remaining new clusters or singletons do not introduce changes to the previously reported by Ribeiro et al., (2019): C9, C19, S1 and S10 fully correspond to Fern, Halcyone, API480 and Lily, respectively.

In most cases, the same cluster comprised prophages from different hosts. The exceptions were C3, C15 and C16, where prophages H11_R10 and H11_R12, H14_R7 and H14_R8 and H9_R14 and H9_R15 share the Host 11 (with 83.8% identity), 14 (with 71.4% identity) and 9 (with 61.4% identity), respectively (**Figure 41** and **Table 24**). Similar phages were found in different hosts, as for example, H11_R15 and H12_R13 (cluster C1–C), H11_R8 and H12_R8 (cluster C20–AU) or H11_R14 and H8_R7 (cluster C7–J).

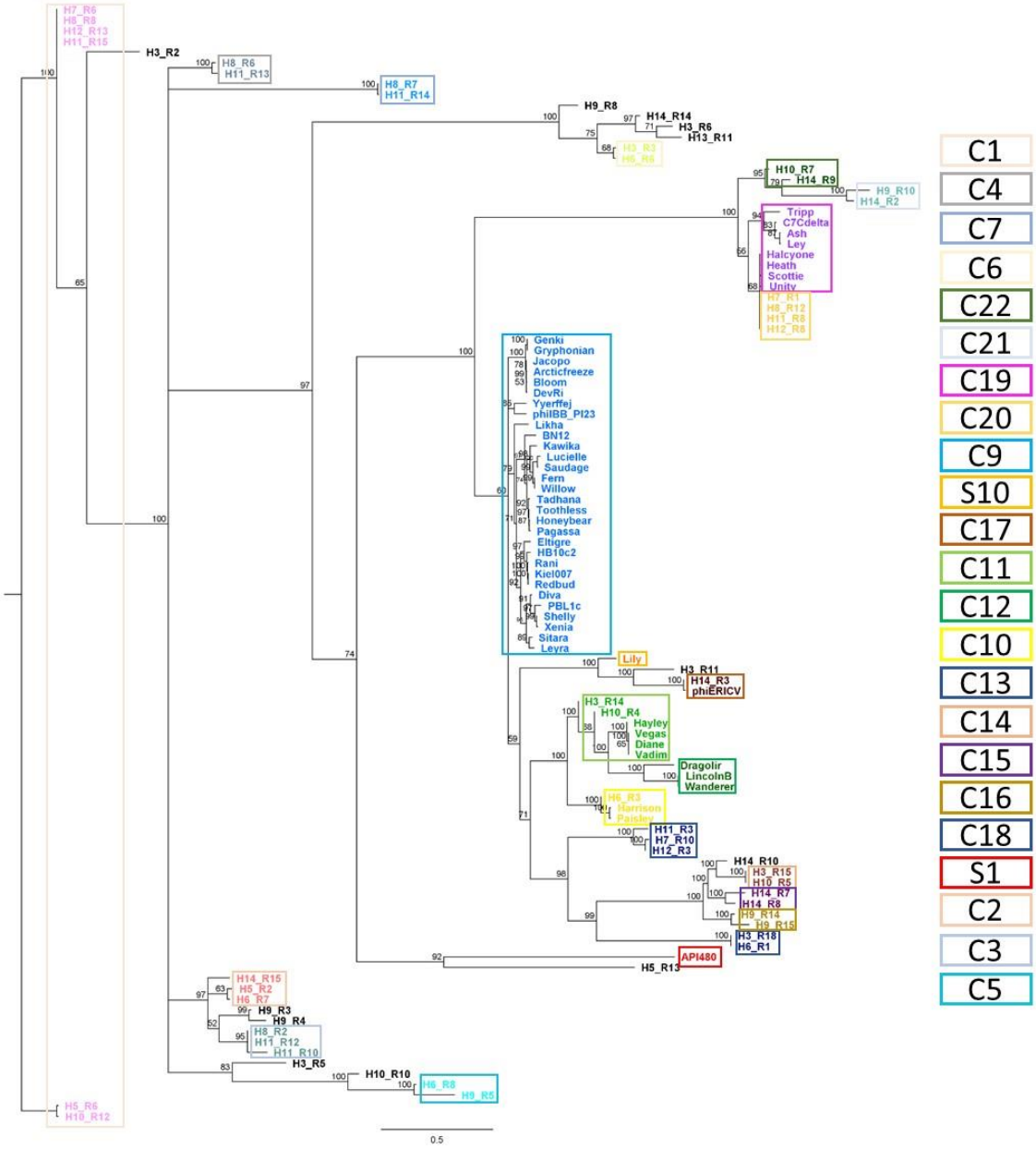

Figure 41: Phylogenetic analysis of *P. larvae* phages. Whole genomes based on shared CDS content (nucleotide), obtained with Geneious. Database: *P. larvae* reported phages (n = 50) and intact *P. larvae* prophages (n = 55), identified here. Clusters have ≥ 60% of shared CDS and were highlighted by coloured rectangles.

Table 24: Shared CDS content. The complete genome sequences of all *P. larvae* phages (n = 50) and prophages identified here (n = 55) were compared through MAFFT and the resulting shared CDS matrix was plotted. Phage clusters: groups with ≥ 60% genome similarity between members; Singletons: < 60% genome similarity with any other phage. Subcluster group of phage with ≥ 90% identity. Cluster division is highlighted with colours.

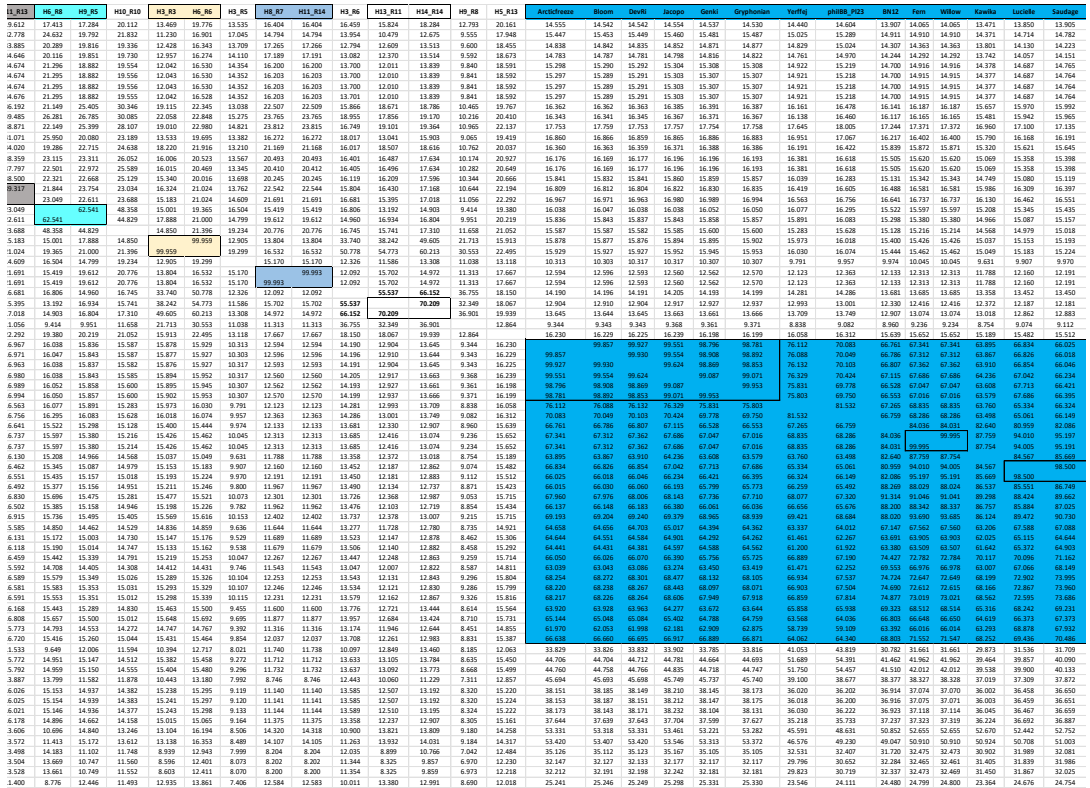

Table 24: *(Continued)*

Honeybear	Toothless	Pagassa	Tadhana	Diva	Shelly	Xenia	Eltigre	HB10c2	Kiel007	Redbud	Rani	Sitara	Leyra	PBL1c	Likha	H6_R3	Harrison	Paisley	Lily	Diane	Vadim	Vegas	Hayley	H3_R14	H10_R4	Dragolir	LincolnB	Wanderer
13.682	14.014	13.657	14.146	13.721	13.518	13.513	13.980	13.237	14.033	14.029	14.052	13.813	13.784	13.096	13.611	12.341	14.338	14.297	11.629	13.379	13.381	13.374	13.164	13.237	13.434	11.371	10.610	10.596
14.637	14.991	14.621	15.082	14.232	14.402	14.416	15.045	14.125	15.176	15.180	15.181	14.553	14.917	13.967	14.925	8.587	14.116	14.125	12.904	13.834	13.835	13.833	13.855	8.583	8.953	13.379	13.459	13.471
14.015	14.351	14.032	14.456	13.666	13.784	13.782	14.452	13.563	14.490	14.487	14.503	14.180	14.327	13.337	14.074	10.364	14.128	14.157	12.038	13.432	13.432	13.432	13.411	10.838	11.192	11.909	11.779	11.784
13.951	14.288	13.960	14.393	13.591	13.725	13.722	14.380	13.492	14.422	14.419	14.435	14.120	14.272	13.270	14.016	10.378	14.170	14.200	11.975	13.429	13.429	13.428	13.407	10.572	10.949	12.145	12.004	12.009
14.558	14.893	14.594	14.985	13.937	14.073	14.063	14.947	13.928	14.993	14.984	15.005	14.427	14.698	13.740	14.624	10.113	14.403	14.423	12.627	14.063	14.065	14.062	14.164	10.603	10.841	12.856	12.956	12.971
14.558	14.893	14.593	14.984	13.936	14.072	14.063	14.947	13.928	14.992	14.983	15.005	14.427	14.698	13.739	14.623	10.113	14.403	14.423	12.626	14.063	14.064	14.062	14.163	10.602	10.841	12.855	12.955	12.970
14.558	14.893	14.593	14.984	13.936	14.072	14.063	14.947	13.928	14.992	14.983	15.005	14.427	14.698	13.739	14.623	10.113	14.403	14.423	12.626	14.063	14.064	14.062	14.163	10.602	10.841	12.855	12.955	12.970
14.558	14.893	14.593	14.984	13.936	14.072	14.063	14.947	13.928	14.992	14.983	15.005	14.427	14.698	13.739	14.623	10.113	14.401	14.421	12.624	14.063	14.064	14.062	14.163	10.602	10.841	12.855	12.953	12.968
15.944	16.250	15.953	16.384	15.596	15.571	15.560	15.848	15.493	16.114	16.107	16.096	15.787	15.983	15.265	15.914	12.240	15.425	15.385	12.110	15.178	15.178	15.176	15.277	16.350	16.338	10.838	10.387	10.403
15.917	16.228	15.925	16.364	15.561	15.537	15.522	15.818	15.455	16.087	16.080	16.071	15.757	15.957	15.222	15.884	12.411	15.401	15.360	15.957	15.142	15.144	15.143	15.250	20.221	19.878	15.343	14.680	14.701
17.059	17.434	17.063	17.605	17.041	16.891	16.888	16.886	16.310	17.207	17.199	17.204	17.514	17.022	16.522	16.904	13.946	17.264	17.298	12.713	16.565	16.567	16.559	16.242	16.288	16.519	11.993	11.222	11.240
16.105	16.402	16.123	16.520	16.014	16.289	16.284	16.161	15.662	16.368	16.358	16.346	16.771	16.634	15.973	16.222	10.673	16.378	16.399	15.167	16.382	16.383	16.379	15.955	10.310	11.123	15.693	14.504	14.525
15.666	15.965	15.667	16.089	15.093	14.949	14.941	15.848	15.401	16.124	16.113	16.125	15.846	15.944	14.663	15.476	12.953	16.109	16.131	11.259	15.512	15.514	15.513	15.531	14.981	15.054	10.865	10.201	10.216
15.345	15.700	15.347	15.835	15.581	15.519	15.502	15.265	14.608	15.472	15.463	15.464	15.787	15.405	15.032	15.275	12.868	15.677	15.693	12.795	14.961	14.963	14.967	14.484	14.306	14.807	12.483	11.552	11.570
15.345	15.700	15.347	15.835	15.581	15.519	15.502	15.265	14.608	15.472	15.463	15.464	15.787	15.405	15.032	15.275	12.811	15.677	15.693	12.479	14.961	14.963	14.967	14.484	14.228	14.769	12.179	11.274	11.292
14.979	15.323	14.981	15.454	15.029	15.081	15.064	15.043	14.214	15.084	15.076	15.093	15.401	15.105	14.686	14.906	13.001	15.572	15.593	12.682	14.702	14.702	14.707	14.222	14.069	14.568	12.342	11.253	11.272
16.342	16.674	16.353	16.756	15.443	15.989	15.975	16.308	15.447	16.435	16.427	16.437	16.029	16.659	15.634	16.567	11.981	15.626	15.646	13.526	15.897	15.896	15.891	16.047	14.605	14.569	12.742	12.740	12.763
16.492	16.830	16.502	16.915	15.585	16.131	16.118	16.459	15.592	16.589	16.581	16.591	16.168	16.808	15.773	16.720	11.533	15.772	15.792	13.887	16.026	16.025	16.021	16.178	13.606	13.572	13.498	13.504	13.528
15.377	15.696	15.385	15.736	14.850	15.172	15.190	15.442	14.708	15.579	15.583	15.553	15.443	15.657	14.793	15.416	9.649	14.951	14.959	13.799	15.153	15.154	15.146	14.896	10.696	11.413	14.183	13.669	13.661
15.156	15.475	15.158	15.495	14.462	15.003	15.014	15.339	14.405	15.349	15.353	15.351	15.289	15.500	14.553	15.260	12.006	15.147	15.150	11.582	14.937	14.939	14.936	14.662	14.840	15.172	11.102	10.747	10.749
14.951	15.281	14.946	15.405	14.529	14.730	14.747	14.791	14.308	15.026	15.031	15.012	14.830	15.012	14.272	15.044	11.594	14.512	14.555	11.878	14.382	14.383	14.377	14.158	13.246	13.612	11.748	11.560	11.552
15.211	15.477	15.198	15.569	14.836	15.147	15.133	15.219	14.412	15.289	15.293	15.298	15.463	15.648	14.747	15.431	10.394	15.382	15.404	10.443	15.238	15.241	15.243	15.015	13.104	13.138	8.939	8.596	8.603
15.246	15.521	15.226	15.616	14.859	15.176	15.162	15.253	14.431	15.326	15.329	15.339	15.500	15.692	14.767	15.464	12.717	15.458	15.480	13.180	15.295	15.297	15.298	15.065	16.194	16.353	12.943	12.401	12.411
9.800	10.073	9.782	10.153	9.636	9.529	9.538	10.047	9.746	10.104	10.107	10.115	9.455	9.695	9.392	9.854	8.021	9.272	9.296	7.992	9.119	9.120	9.133	9.164	8.506	8.489	7.999	8.073	8.070
11.967	12.301	11.962	12.402	11.644	11.689	11.679	12.267	11.543	12.253	12.246	12.231	11.600	11.877	11.316	12.037	11.740	11.712	11.732	8.746	11.140	11.141	11.144	11.375	14.320	14.107	8.204	8.202	8.200
11.967	12.301	11.962	12.402	11.644	11.689	11.679	12.267	11.543	12.253	12.246	12.231	11.600	11.877	11.316	12.037	11.738	11.712	11.732	8.746	11.140	11.141	11.144	11.375	14.318	14.105	8.204	8.202	8.200
13.490	13.726	13.476	13.737	13.277	13.523	13.506	13.447	13.047	13.543	13.534	13.579	13.776	13.957	13.174	13.708	10.097	13.633	13.637	12.443	13.585	13.585	13.589	13.358	10.900	11.263	12.035	11.344	11.354
12.134	12.368	12.103	12.378	11.728	12.147	12.140	12.248	12.007	12.131	12.121	12.162	12.721	12.684	11.946	12.261	12.849	13.105	13.092	10.060	12.507	12.507	12.510	12.237	13.821	13.932	8.899	8.325	8.325
12.737	12.987	12.719	13.007	12.780	12.878	12.882	12.863	12.822	12.843	12.830	12.867	13.444	13.424	12.644	12.983	13.460	13.784	13.773	11.229	13.192	13.192	13.195	12.907	13.809	14.031	10.766	9.857	9.859
8.871	9.053	8.854	9.215	8.735	8.462	8.458	9.259	8.587	9.296	9.286	9.326	8.614	8.710	8.451	8.831	8.185	8.635	8.668	7.311	8.320	8.320	8.324	8.305	9.180	9.184	7.042	6.970	6.973
15.423	15.715	15.434	15.715	14.921	15.306	15.292	15.714	14.811	15.804	15.799	15.816	15.564	15.731	14.855	15.387	12.063	15.450	15.499	12.857	15.220	15.224	15.222	15.161	14.258	14.317	12.484	12.230	12.218
66.015	67.960	66.137	69.193	64.658	64.644	64.441	66.050	63.039	68.254	68.220	68.217	63.920	65.144	61.970	66.638	33.829	44.706	44.760	45.694	38.151	38.153	38.173	37.644	53.331	53.420	35.126	32.147	32.212
66.030	67.976	66.148	69.204	64.656	64.551	64.431	66.026	63.043	68.272	68.238	68.226	63.928	65.048	62.053	66.660	33.826	44.704	44.758	45.693	38.185	38.187	38.143	37.639	53.318	53.407	35.112	32.127	32.191
66.060	68.006	66.183	69.240	64.703	64.584	64.381	66.070	63.086	68.301	68.267	68.264	63.963	65.084	61.998	66.695	33.832	44.712	44.766	45.698	38.149	38.151	38.171	37.643	53.331	53.420	35.123	32.133	32.198
66.193	68.143	66.380	69.379	65.017	64.901	64.597	66.390	63.274	68.477	68.443	68.606	64.277	65.402	62.181	66.917	33.902	44.781	44.835	45.749	38.210	38.212	38.232	37.704	53.461	53.546	35.167	32.177	32.242
65.799	67.736	66.061	68.965	64.394	64.292	64.588	65.756	63.450	68.132	68.097	67.949	63.672	64.788	62.909	66.889	33.785	44.664	44.718	45.737	38.145	38.147	38.104	37.599	53.221	53.313	35.105	32.117	32.181
65.773	67.710	66.036	68.939	64.362	64.262	64.562	65.725	63.419	68.105	68.071	67.918	63.644	64.759	62.875	66.871	33.816	44.693	44.747	45.740	38.173	38.175	38.131	37.627	53.282	53.372	35.105	32.117	32.181
66.259	68.077	66.656	69.421	63.337	61.461	61.200	66.889	61.471	66.934	66.903	66.859	65.858	63.568	58.739	64.062	41.053	51.689	51.750	39.100	36.020	36.018	36.030	35.218	45.591	46.576	32.531	29.796	29.823
65.492	67.320	65.676	68.684	64.012	62.267	61.922	67.190	62.252	67.537	67.504	67.814	65.938	64.036	59.109	64.340	43.819	54.391	54.457	38.677	36.202	36.200	36.222	35.733	48.631	49.230	32.407	30.652	30.719
88.269	91.314	88.200	88.020	67.147	63.691	63.380	74.427	69.553	74.724	74.690	74.877	69.323	66.803	63.392	68.803	30.782	41.462	41.510	38.377	36.914	36.916	36.923	37.237	50.852	49.047	31.720	32.284	32.337
88.029	91.046	88.342	93.690	67.562	63.905	63.509	72.782	66.976	72.647	72.612	73.019	68.512	66.648	66.016	71.552	31.661	41.962	42.012	38.327	37.074	37.075	37.118	37.323	52.655	50.910	32.475	32.465	32.473
88.024	91.041	88.337	93.685	67.560	63.903	63.507	72.784	66.978	72.649	72.615	73.021	68.514	66.650	66.014	71.547	31.661	41.962	42.012	38.328	37.070	37.071	37.114	37.319	52.655	50.910	32.473	32.461	32.469
86.537	89.298	86.757	86.124	63.206	62.025	61.642	70.117	63.007	68.199	68.166	68.562	65.316	64.619	63.293	68.252	29.873	39.464	39.538	37.019	36.002	36.003	36.045	36.224	52.670	50.924	30.902	31.405	31.450
85.551	88.424	85.884	89.472	67.588	65.115	65.372	70.096	67.066	72.902	72.867	72.595	68.242	66.373	68.878	69.436	31.536	39.857	39.900	37.309	36.458	36.459	36.467	36.692	52.442	50.708	31.989	31.839	31.867
86.749	89.662	87.025	90.730	67.088	64.644	64.903	71.162	68.149	73.995	73.960	73.686	69.231	67.373	67.932	70.486	31.709	40.090	40.133	37.872	36.650	36.651	36.659	36.887	52.752	51.003	32.081	31.986	32.025
	96.531	99.267	93.245	66.787	63.372	63.216	73.028	68.319	73.732	73.699	73.490	69.241	66.327	64.611	70.473	30.289	42.039	42.088	38.142	36.657	36.659	36.674	36.897	52.592	50.851	31.745	32.236	32.270
96.531		95.845	96.496	68.793	65.208	65.048	74.960	70.358	75.674	75.640	75.429	70.896	67.927	66.541	72.412	31.075	41.814	41.863	39.080	37.593	37.595	37.610	37.854	52.597	50.855	32.468	32.929	32.964
99.267	95.845		92.518	67.060	63.567	63.362	73.331	68.439	74.217	74.184	73.781	69.413	66.520	64.723	70.636	30.316	42.063	42.112	38.135	36.674	36.676	36.683	36.906	52.673	50.926	31.696	32.199	32.233
93.245	96.496	92.518		70.403	65.657	65.495	73.790	70.795	76.327	76.293	76.074	71.425	68.419	66.985	73.578	31.733	42.659	42.710	39.655	37.672	37.674	37.690	37.938	52.610	50.868	33.115	32.886	32.921
66.787	68.793	67.060	70.403		86.120	85.731	66.704	69.250	70.975	70.942	71.194	80.470	73.649	76.972	66.129	33.787	39.650	39.718	36.519	34.195	34.197	34.212	33.685	46.515	46.817	31.745	28.936	28.982
63.372	65.208	63.567	65.657	86.120		99.512	64.614	63.394	69.009	68.977	69.409	79.651	86.310	87.779	76.316	31.121	40.749	40.815	42.955	35.071	35.073	35.114	34.585	46.502	46.805	30.903	30.460	30.505
63.216	65.048	63.362	65.495	85.731	99.512		64.248	63.660	68.784	68.752	69.004	79.256	85.899	88.130	76.433	31.069	40.707	40.773	42.959	35.079	35.081	35.037	34.552	46.407	46.712	30.896	30.453	30.498
73.028	74.960	73.331	73.790	66.704	64.614	64.248		85.236	91.585	91.549	91.783	76.725	76.871	63.658	69.799	31.027	41.253	41.302	37.208	37.102	37.106	37.125	37.667	53.055	50.468	31.995	32.708	32.759
68.319	70.358	68.439	70.795	69.250	63.394	63.660	85.236		91.541	91.504	91.310	75.988	74.684	61.110	66.750	33.034	38.997	39.043	36.026	35.634	35.636	35.646	36.177	52.905	50.329	31.599	30.672	30.726
73.732	75.674	74.217	76.327	70.975	69.009	68.784	91.585	91.541		99.958	99.447	82.952	81.787	66.187	72.285	31.566	41.496	41.545	38.825	37.282	37.284	37.294	37.853	53.084	50.496	32.670	32.667	32.722
73.699	75.640	74.184	76.293	70.942	68.977	68.752	91.549	91.504	99.958		99.410	82.919	81.754	66.155	72.252	31.533	41.465	41.514	38.810	37.255	37.257	37.267	37.826	53.022	50.437	32.663	32.660	32.714
73.490	75.429	73.781	76.074	71.194	69.409	69.004	91.783	91.310	99.447	99.410		83.317	82.183	66.031	72.197	31.544	41.466	41.515	38.859	37.231	37.233	37.278	37.805	53.041	50.456	32.653	32.641	32.695
69.241	70.896	69.413	71.425	80.470	79.651	79.256	76.725	75.988	82.952	82.919	83.317		85.026	71.224	68.229	32.542	41.852	41.899	37.980	35.803	35.805	35.847	35.327	47.202	47.487	31.467	30.239	30.283
66.327	67.927	66.520	68.419	73.649	86.310	85.899	76.871	74.684	81.787	81.754	82.183	85.026		75.421	80.955	31.535	41.709	41.784	43.805	36.502	36.504	36.547	36.968	50.232	48.123	32.172	31.848	31.893
64.611	66.541	64.723	66.985	76.972	87.779	88.130	63.658	61.110	66.187	66.155	66.031	71.224	75.421		71.999	29.609	38.682	38.745	42.442	35.088	35.091	35.048	34.556	48.546	48.823	30.605	30.561	30.617
70.473	72.412	70.636	73.578	66.129	76.316	76.433	69.799	66.750	72.285	72.252	72.197	68.229	80.955	71.999		30.277	41.869	41.915	44.556	36.561	36.563	36.607	37.102	52.074	49.645	32.075	32.416	32.424
30.289	31.075	30.316	31.733	33.787	31.121	31.069	31.027	33.034	31.566	31.533	31.544	32.542	31.535	29.609	30.277		64.381	64.487	17.901	38.312	38.314	38.322	37.619	49.509	50.485	25.243	22.060	22.112
42.039	41.814	42.063	42.659	39.650	40.749	40.707	41.253	38.997	41.496	41.465	41.466	41.852	41.709	38.682	41.869	64.381		98.711	30.379	43.484	43.485	43.484	42.876	68.822	69.845	37.056	34.727	34.791
42.088	41.863	42.112	42.710	39.718	40.815	40.773	41.302	39.043	41.545	41.514	41.515	41.899	41.784	38.745	41.915	64.487	98.711		30.425	43.565	43.567	43.565	42.956	68.831	69.847	37.119	34.746	34.810
38.142	39.080	38.135	39.655	36.519	42.955	42.959	37.208	36.026	38.825	38.810	38.859	37.980	43.805	42.442	44.556	17.901	30.379	30.425		27.100	27.100	27.100	26.590	18.890	20.084	27.424	24.378	24.472
36.657	37.593	36.674	37.672	34.195	35.071	35.079	37.102	35.634	37.282	37.255	37.231	35.803	36.502	35.088	36.561	38.312	43.484	43.565	27.100		99.989	99.935	96.825	92.012	99.828	57.587	48.389	48.329
36.659	37.595	36.676	37.674	34.197	35.073	35.081	37.106	35.636	37.284	37.257	37.233	35.805	36.504	35.091	36.563	38.314	43.485	43.567	27.100	99.989		99.942	96.832	92.017	99.832	57.590	48.390	48.330
36.674	37.610	36.683	37.690	34.212	35.114	35.037	37.125	35.646	37.294	37.267	37.278	35.847	36.547	35.048	36.607	38.322	43.484	43.565	27.100	99.935	99.942		96.828	92.035	99.851	57.600	48.402	48.342
36.897	37.854	36.906	37.938	33.685	34.585	34.552	37.667	36.177	37.853	37.826	37.805	35.327	36.968	34.556	37.102	37.619	42.876	42.956	26.590	96.825	96.832	96.828		98.600	93.493	57.137	49.325	49.264
52.592	52.597	52.673	52.610	46.515	46.502	46.407	53.055	52.905	53.084	53.022	53.041	47.202	50.232	48.546	52.074	49.509	68.822	68.831	18.890	92.012	92.017	92.035	98.600		78.811	29.771	29.146	29.085
50.851	50.855	50.926	50.868	46.817	46.805	46.712	50.468	50.329	50.496	50.437	50.456	47.487	48.123	48.823	49.645	50.485	69.845	69.847	20.084	99.828	99.832	99.851	93.493	78.811		31.477	29.643	29.582
31.745	32.468	31.696	33.115	31.745	30.903	30.896	31.995	31.599	32.670	32.663	32.653	31.467	32.172	30.605	32.075	25.243	37.056	37.119	27.424	57.587	57.590	57.600	57.137	29.771	31.477		59.574	59.561
32.236	32.929	32.199	32.886	28.936	30.460	30.453	32.708	30.672	32.667	32.660	32.641	30.239	31.848	30.561	32.416	22.060	34.727	34.746	24.378	48.389	48.390	48.402	49.325	29.146	29.643	59.574		99.728
32.270	32.964	32.233	32.921	28.982	30.505	30.498	32.759	30.726	32.722	32.714	32.695	30.283	31.893	30.617	32.424	22.112	34.791	34.810	24.472	48.329	48.330	48.342	49.264	29.085	29.582	59.561	99.728	
23.946	24.604	23.934	25.008	24.330	23.189	23.201	23.843	24.070	24.319	24.309	24.317	22.843	23.173	23.033	23.811	22.416	21.854	21.910	27.205	20.968	20.968	20.970	21.087	31.716	30.627	13.202	12.498	12.637
33.302	34.215	33.310	34.636	33.999	33.271	33.239	33.341	33.116	33.964	33.960	33.940	32.703	33.394	32.118	33.696	25.583	26.106	26.202	18.062	25.558	25.562	25.558	25.502	29.440	29.484	17.685	17.167	17.205
33.994	35.019	34.021	35.499	34.868	35.412	35.369	34.106	33.880	34.807	34.803	34.788	34.513	35.515	34.106	35.952	26.791	26.619	26.723	18.283	26.402	26.406	26.406	26.338	29.431	29.486	17.592	17.052	17.094
33.035	33.968	33.035	34.399	33.580	32.664	32.622	33.139	32.809	33.800	33.796	33.791	32.589	32.976	31.465	33.209	27.561	25.983	26.039	17.959	25.001	25.003	25.005	24.903	30.490	30.569	18.251	17.463	17.504
21.158	21.686	21.147	21.921	20.187	20.177	20.176	21.374	20.361	21.590	21.580	21.594	20.226	20.767	20.098	21.093	21.661	21.384	21.427	15.619	20.255	20.257	20.262	20.483	23.243	23.098	15.164	15.219	15.256
21.158	21.686	21.147	21.921	20.187	20.177	20.176	21.374	20.361	21.590	21.580	21.594	20.226	20.767	20.098	21.093	21.658	21.382	21.425	15.617	20.253	20.255	20.261	20.481	23.243	23.100	15.165	15.221	15.257
15.959	16.366	15.946	16.619	16.423	15.548	15.547	16.355	16.695	16.680	16.669	16.675	14.972	15.328	15.661	16.097	22.871	16.129	16.151	12.141	15.623	15.624	15.628	15.973	23.338	23.009	12.090	12.253	12.294
21.460	21.985	21.419	22.244	20.476	19.874	19.889	22.011	21.532	22.234	22.224	22.233	20.791	20.861	19.740	21.024	22.910	21.337	21.385	16.438	20.524	20.526	20.532	20.552	23.904	23.880	16.332	15.508	15.542
18.034	18.500	18.001	18.846	18.314	17.191	17.206	18.502	19.299	18.792	18.781	18.791	17.057	17.433	17.216	17.986	23.421	17.867	17.889	13.822	17.341	17.343	17.347	17.614	23.408	23.083	13.804	13.378	13.454
18.611	19.059	18.573	19.307	18.607	17.868	17.879	18.517	18.306	18.679	18.668	18.682	17.944	18.235	17.502	18.476	23.201	19.792	19.821	13.455	18.174	18.176	18.183	18.291	23.407	23.117	13.514	12.990	13.010
22.749	23.255	22.728	23.546	22.926	22.724	22.731	22.328	22.708	22.934	22.925	22.920	22.201	23.017	22.508	23.427	19.070	21.529	21.532	17.136	21.784	21.786	21.794	21.967	23.651	23.433	17.580	17.030	17.050
21.742	22.436	21.725	22.983	22.698	22.084	22.106	21.822	22.684	22.353	22.341	22.354	20.886	21.698	22.841	22.844	20.698	19.479	19.504	30.220	19.927	19.927	19.930	20.425	24.945	24.159	14.083	13.804	13.835
21.164	21.729	21.152	22.169	21.932	20.628	20.645	21.176	21.882	21.639	21.629	21.639	19.804	20.344	20.683	21.207	19.914	19.091	19.110	27.156	18.533	18.533	18.535	18.906	24.998	24.175	13.950	13.651	13.678
24.861	25.529	24.857	25.920	23.724	23.051	23.052	24.803	24.385	25.162	25.152	25.162	23.731	24.063	23.173	24.700	19.938	22.902	22.969	18.160	22.823	22.825	22.835	22.895	26.167	33.870	18.103	17.346	17.334
24.853	25.521	24.850	25.912	23.718	23.046	23.047	24.798	24.379	25.157	25.146	25.157	23.726	24.058	23.168	24.693	19.934	22.894	22.961	18.160	22.819	22.820	22.830	22.890	26.167	33.871	18.100	17.343	17.331
20.502	20.493	20.528	20.484	19.829	19.843	19.808	20.074	20.056	20.057	20.048	20.068	20.186	19.913	20.413	20.047	19.781	21.856	21.857	13.596	22.591	22.595	22.607	22.098	21.106	21.202	12.231	11.361	11.366
20.498	20.488	20.524	20.479	19.825	19.839	19.804	20.069	20.052	20.053	20.044	20.063	20.182	19.909	20.409	20.043	19.777	21.836	21.838	13.594	22.579	22.582	22.595	22.086	21.113	21.220	12.234	11.348	11.353
20.728	20.719	20.755	20.713	20.047	20.061	20.025	20.319	20.300	20.302	20.292	20.312	20.409	20.137	20.668	20.269	19.925	22.125	22.126	13.692	22.869	22.872	22.885	22.372	21.241	21.379	12.302	11.415	11.427
20.753	20.743	20.764	20.734	20.563	20.572	20.559	20.706	20.710	20.679	20.673	20.700	20.941	20.873	20.367	20.674	20.094	22.961	22.962	13.377	23.047	23.051	23.051	22.648	21.134	21.135	12.086	11.046	11.051
20.751	20.742	20.763	20.732	20.561	20.570	20.557	20.705	20.708	20.678	20.671	20.698	20.939	20.871	20.365	20.673	20.090	22.956	22.958	13.382	23.043	23.046	23.046	22.643	21.131	21.132	12.085	11.045	11.050
20.754	20.745	20.766	20.735	20.564	20.573	20.560	20.708	20.712	20.681	20.675	20.702	20.942	20.874	20.368	20.676	19.665	22.944	22.946	13.130	23.033	23.036	23.036	22.633	20.613	20.560	11.896	10.868	10.873
20.839	20.829	20.857	20.819	20.649	20.658	20.645	20.800	20.803	20.773	20.766	20.793	21.028	20.963	20.461	20.753	20.041	23.007	23.008	14.758	23.097	23.101	23.101	22.697	20.696	20.818	13.298	12.184	12.190
18.858	18.855	18.893	18.842	18.300	18.292	18.272	18.192	18.194	18.191	18.182	18.189	18.847	18.525	18.549	18.317	19.333	21.529	21.531	12.106	20.682	20.685	20.692	20.136	19.949	19.998	10.974	9.687	9.697
21.605	22.000	21.614	22.086	20.378	21.607	21.602	21.738	20.355	21.980	21.977	21.977	21.634	22.278	21.188	22.124	14.380	20.648	20.646	19.775	21.047	21.049	21.050	20.833	18.898	19.741	19.156	18.739	18.743
21.557	21.949	21.566	22.034	20.336	21.558	21.555	21.686	20.307	21.928	21.925	21.922	21.585	22.225	21.141	22.068	12.730	20.626	20.624	19.739	21.003	21.004	21.003	20.786	14.543	15.280	19.009	18.595	18.599
21.557	21.949	21.565	22.034	20.335	21.557	21.555	21.686	20.306	21.928	21.925	21.922	21.584	22.224	21.140	22.068	12.730	20.625	20.623	19.738	21.002	21.004	21.002	20.786	14.543	15.280	19.009	18.595	18.599
21.557	21.949	21.565	22.034	20.335	21.557	21.555	21.686	20.306	21.928	21.925	21.922	21.584	22.224	21.140	22.068	12.730	20.625	20.623	19.738	21.002	21.004	21.002	20.786	14.543	15.280	19.009	18.595	18.599
15.942	16.286	15.928	16.281	14.407	15.578	15.574	16.574	14.859	16.849	16.847	16.853	16.144	16.501	15.059	16.228	7.766	14.099	14.093	13.675	14.921	14.921	14.923	14.811	8.278	8.617	13.108	12.981	12.985
16.438	16.782	16.433	16.799	14.828	15.963	15.958	16.939	15.206	17.230	17.228	17.222	16.500	16.835	15.445	16.526	7.559	14.567	14.554	14.339	15.216	15.216	15.218	15.095	7.691	8.179	13.806	13.670	13.667
20.326	20.695	20.336	20.730	18.866	19.828	19.830	19.863	18.474	20.065	20.058	20.067	20.048	20.492	19.692	20.451	11.972	19.487	19.477	18.894	19.109	19.111	19.119	18.985	12.686	13.247	17.882	17.407	17.417
21.414	21.788	21.422	21.841	20.057	21.033	21.040	21.372	20.074	21.655	21.650	21.644	21.432	21.999	20.645	21.807	13.967	20.710	20.700	19.229	20.726	20.727	20.732	20.658	16.243	16.703	17.928	17.501	17.512

Table 24: *(Continued)*

H3_R11	H7_R10	H12_R3	H11_R3	H3_R15	H10_R5	H14_R10	H14_R7	H14_R8	H9_R14	H9_R15	H14_R3	phiERICV	H3_R18	H6_R1	Ash	Ley	C7Cdelta	Halcyone	Heath	Scottie	Unity	Tripp	H7_R1	H8_R12	H11_R8	H12_R8	H9_R10	H14_R2	H10_R7	H14_R9	Phage	Clusters/Singletons	Subcluster
11.489	12.099	12.358	13.180	12.231	12.228	11.052	12.634	11.574	12.098	12.774	12.045	12.081	12.501	12.498	12.997	13.006	13.074	12.972	12.976	12.873	12.817	12.148	12.889	12.341	12.341	12.341	8.408	8.286	10.959	12.440	API480	S1	
7.572	9.556	9.358	10.310	9.478	9.478	7.538	9.492	8.146	8.485	10.390	8.180	8.486	10.073	10.076	8.025	8.008	8.065	7.995	8.001	7.900	8.737	7.591	12.712	12.452	12.452	12.452	9.967	10.391	12.375	12.015	H3_R2	S2	
9.381	11.190	11.197	11.329	10.520	10.521	8.802	10.700	9.257	10.111	10.672	9.617	9.536	10.873	10.877	10.102	10.102	10.141	10.138	10.133	9.808	10.938	9.837	12.456	11.369	11.369	11.369	9.423	9.787	11.721	11.950	H5_R6	C1	A
9.226	11.284	11.318	11.329	10.529	10.531	8.742	10.623	9.239	10.018	10.628	9.425	9.407	10.904	10.908	9.879	9.879	9.918	9.949	9.944	9.758	10.788	9.586	12.434	11.701	11.701	11.701	9.587	9.978	11.931	12.081	H10_R12	C1	A
9.064	10.629	10.619	11.508	10.405	10.405	8.914	10.430	9.187	9.823	11.115	9.465	9.565	10.995	10.996	9.927	9.924	9.961	9.942	9.942	9.822	10.724	9.128	12.877	12.241	12.241	12.241	9.156	9.445	12.249	12.238	H7_R6	C1	B
9.064	10.630	10.620	11.509	10.407	10.407	8.915	10.431	9.189	9.825	11.116	9.464	9.564	10.996	10.997	9.927	9.923	9.961	9.941	9.941	9.822	10.724	9.128	12.876	12.240	12.240	12.240	9.157	9.444	12.250	12.239	H8_R8	C1	B
9.064	10.630	10.620	11.509	10.409	10.409	8.915	10.431	9.189	9.825	11.116	9.464	9.564	10.996	10.997	9.925	9.922	9.959	9.940	9.940	9.820	10.722	9.126	12.876	12.240	12.240	12.240	9.157	9.444	12.250	12.239	H12_R13	C1	B
9.064	10.630	10.620	11.509	10.407	10.407	8.915	10.431	9.189	9.825	11.116	9.464	9.565	10.997	10.998	9.927	9.923	9.961	9.941	9.941	9.822	10.724	9.128	12.876	12.240	12.240	12.240	9.157	9.443	12.249	12.239	H11_R15	C1	B
13.456	13.415	14.683	14.015	12.775	12.776	11.402	12.759	11.897	12.732	14.395	13.675	12.959	12.707	12.707	15.097	15.098	15.181	15.166	15.169	14.820	14.667	14.295	15.034	12.274	12.274	12.274	8.840	8.505	11.407	13.500	H5_R2	C2	C
12.823	14.211	15.319	14.139	13.437	13.437	10.983	13.178	12.170	12.789	14.598	14.479	13.305	13.507	13.508	16.706	16.691	16.898	17.417	17.422	17.428	17.510	15.601	16.457	14.644	14.644	14.644	10.377	10.628	13.473	16.280	H6_R7	C2	C
13.924	14.716	14.846	15.104	14.039	14.038	12.083	14.362	12.719	13.578	14.707	13.672	13.235	13.538	13.536	15.176	15.181	15.324	15.408	15.405	15.003	15.264	14.352	15.078	12.876	12.875	12.875	9.613	9.428	12.109	13.765	H14_R15	C2	D
8.559	10.791	10.533	11.293	10.311	10.313	8.113	10.936	9.104	9.403	12.233	9.562	9.570	11.183	11.183	9.979	9.969	10.015	10.082	10.085	9.974	10.880	9.149	15.327	15.189	15.189	15.189	10.024	10.602	14.196	14.042	H9_R3	S3	
13.289	13.037	13.936	13.800	12.729	12.731	10.947	13.327	11.655	12.663	13.791	12.752	12.385	12.560	12.560	14.522	14.527	14.553	14.516	14.512	14.244	13.910	13.594	14.067	12.168	12.168	12.168	8.821	8.536	11.049	13.065	H9_R4	S4	
12.096	13.320	13.457	13.497	12.739	12.738	10.986	12.969	11.436	12.120	13.167	12.454	11.899	12.772	12.771	13.412	13.412	13.512	13.652	13.657	13.267	13.784	12.451	14.032	12.692	12.692	12.692	9.382	9.316	12.226	13.306	H8_R2	C3	E
12.098	13.255	13.386	13.423	12.668	12.667	10.960	12.915	11.407	12.054	13.257	12.434	11.890	12.770	12.770	13.376	13.376	13.474	13.580	13.585	13.203	13.557	12.439	13.958	12.647	12.646	12.646	9.175	9.094	11.965	13.080	H11_R12	C3	E
11.734	12.296	12.954	13.356	12.596	12.595	10.955	12.882	11.394	12.240	12.980	12.097	11.674	12.587	12.587	13.180	13.179	13.277	13.389	13.394	13.053	13.504	12.284	13.790	12.595	12.595	12.595	9.198	9.100	12.022	13.096	H11_R10	C3	F
12.294	13.428	13.547	13.757	12.987	12.990	10.992	13.130	11.581	12.195	13.865	12.506	12.170	12.869	12.872	13.566	13.565	13.638	13.601	13.601	13.331	13.876	12.778	14.845	13.168	13.168	13.168	10.085	9.914	12.661	14.104	H8_R6	C4	G
11.400	12.783	12.771	13.269	12.250	12.252	10.448	12.497	10.965	11.585	13.895	12.041	11.841	12.691	12.694	12.751	12.750	12.819	12.731	12.732	12.522	12.952	11.903	15.060	14.043	14.043	14.043	10.150	10.124	13.231	14.202	H11_R13	C4	G
8.776	11.546	11.474	11.410	10.023	10.022	7.521	10.552	8.609	8.985	11.792	9.784	9.418	11.242	11.241	10.084	10.082	10.141	10.266	10.272	10.104	10.788	9.716	14.069	14.043	14.042	14.042	10.823	11.041	13.476	13.600	H6_R8	C5	H
12.446	13.448	13.587	13.605	12.250	12.249	10.048	12.603	10.872	11.963	13.291	11.573	11.104	12.326	12.325	13.813	13.815	13.896	14.025	14.031	13.694	14.009	13.560	14.061	11.939	11.939	11.939	10.152	9.759	11.801	13.102	H9_R5	C5	H
11.493	12.614	12.715	13.241	11.899	11.900	10.448	12.382	11.100	11.308	13.093	12.429	12.094	12.589	12.589	12.698	12.701	12.774	12.673	12.680	12.488	12.361	12.214	13.340	12.696	12.695	12.695	9.735	9.432	11.634	13.130	H10_R10	S5	
12.935	11.781	12.229	12.206	11.233	11.236	9.226	11.008	9.580	10.935	13.764	10.721	10.596	10.654	10.654	12.940	12.952	13.021	12.975	12.975	12.672	12.530	12.448	15.040	10.333	10.333	10.333	7.439	7.197	9.744	11.318	H3_R3	C6	I
13.861	14.276	14.239	14.204	13.587	13.588	10.940	13.762	11.885	12.739	14.908	13.778	13.175	13.725	13.725	15.774	15.795	15.925	15.745	15.746	15.551	15.099	14.901	15.189	15.197	15.197	15.197	10.123	9.930	13.485	15.312	H6_R6	C6	I
7.406	8.156	8.142	8.739	7.953	7.955	7.617	8.248	8.101	7.453	8.254	8.675	8.510	8.627	8.624	7.568	7.573	7.668	7.314	7.311	7.241	7.294	7.504	7.967	7.878	7.878	7.878	6.786	6.874	7.690	7.982	H3_R5	S6	
12.584	12.096	12.831	12.848	11.685	11.687	11.159	11.875	11.380	11.975	11.531	12.599	12.124	11.899	11.897	12.894	12.898	13.008	13.053	13.059	12.710	12.522	12.834	10.696	9.352	9.352	9.352	8.004	7.575	8.990	10.662	H8_R7	C7	J
12.583	12.095	12.829	12.847	11.685	11.687	11.157	11.876	11.381	11.977	11.531	12.600	12.124	11.899	11.896	12.892	12.896	13.006	13.052	13.057	12.708	12.520	12.832	10.695	9.352	9.352	9.352	8.003	7.574	8.990	10.662	H11_R14	C7	J
10.011	10.720	10.690	11.243	9.877	9.875	8.474	10.716	9.290	9.580	12.783	11.112	10.735	10.935	10.939	11.331	11.329	11.379	11.279	11.279	11.220	10.631	10.390	14.473	14.385	14.385	14.385	8.611	8.483	11.918	13.203	H3_R6	S7	
13.380	12.767	12.768	12.793	12.233	12.231	11.280	12.648	11.768	12.442	12.808	12.400	12.306	11.542	11.547	14.213	14.210	14.343	14.240	14.245	13.954	13.709	13.332	12.827	10.890	10.890	10.890	6.981	6.717	9.629	11.993	H13_R11	C8	K
12.991	13.009	13.011	13.026	12.251	12.251	11.479	13.068	12.165	12.683	13.306	12.581	12.576	12.347	12.350	14.295	14.300	14.375	14.364	14.367	14.209	13.685	13.264	13.655	13.090	13.089	13.089	7.548	7.179	10.819	13.020	H14_R14	C8	K
8.690	8.828	9.115	8.859	8.254	8.253	8.479	8.913	8.707	8.797	8.974	9.748	9.304	8.778	8.780	8.781	8.792	8.877	8.529	8.529	8.456	7.618	8.346	8.836	8.698	8.698	8.698	6.247	5.874	7.031	8.084	H9_R8	S8	
12.018	13.502	13.633	14.072	12.750	12.749	10.885	13.044	11.505	12.189	13.892	12.350	12.238	13.167	13.169	13.744	13.750	13.817	13.804	13.803	13.543	13.726	13.094	14.679	13.555	13.555	13.555	10.389	10.330	12.734	14.263	H5_R13	S9	
25.241	34.648	35.955	34.462	21.854	21.852	16.391	22.454	18.745	19.335	23.676	22.539	21.691	25.798	25.792	21.166	21.162	21.435	21.757	21.755	21.758	21.834	19.655	22.490	22.439	22.438	22.438	17.178	17.430	21.039	22.371	Arcticfreeze	C9	L
25.246	34.654	35.959	34.473	21.851	21.849	16.384	22.445	18.734	19.324	23.687	22.540	21.697	25.785	25.779	21.157	21.153	21.425	21.760	21.758	21.762	21.837	19.643	22.505	22.453	22.452	22.452	17.185	17.439	21.049	22.387	Bloom	C9	L
25.249	34.649	35.953	34.465	21.853	21.851	16.393	22.453	18.742	19.334	23.694	22.539	21.696	25.795	25.789	21.166	21.162	21.435	21.757	21.755	21.758	21.834	19.655	22.499	22.447	22.447	22.447	17.188	17.441	21.049	22.386	DevRi	C9	L
25.298	34.729	36.040	34.544	21.906	21.904	16.449	22.489	18.798	19.366	23.722	22.609	21.755	25.809	25.803	21.210	21.206	21.479	21.780	21.778	21.782	21.877	19.700	22.519	22.467	22.466	22.466	17.199	17.452	21.066	22.414	Jacopo	C9	L
25.331	34.589	35.885	34.405	21.885	21.883	16.428	22.467	18.779	19.338	23.713	22.643	21.783	25.828	25.822	21.163	21.159	21.431	21.727	21.726	21.729	21.831	19.652	22.499	22.446	22.445	22.445	17.185	17.440	21.053	22.399	Genki	C9	L
25.330	34.588	35.885	34.404	21.877	21.875	16.417	22.456	18.767	19.325	23.701	22.648	21.787	25.828	25.822	21.166	21.162	21.434	21.727	21.726	21.729	21.831	19.646	22.499	22.445	22.445	22.445	17.193	17.446	21.050	22.395	Gryphonian	C9	L
23.546	32.738	33.367	32.593	20.777	20.775	15.093	21.131	17.294	18.585	22.412	20.518	20.214	24.119	24.114	20.157	20.154	20.356	20.962	20.961	20.964	21.052	19.458	22.071	22.019	22.019	22.019	16.319	16.738	20.945	22.071	Yerffej	C9	M
24.111	34.036	34.812	33.726	21.636	21.634	16.005	21.321	17.662	19.319	23.258	21.743	21.196	24.869	24.862	21.208	21.188	21.476	22.157	22.155	22.155	22.250	20.559	22.364	22.311	22.311	22.311	16.434	16.859	21.091	22.269	philBB_Pl23	C9	N
24.480	33.626	34.366	33.380	21.405	21.405	16.206	21.569	18.192	18.604	22.799	22.014	21.384	24.821	24.814	20.058	20.054	20.297	20.330	20.328	20.331	20.419	18.472	21.764	21.715	21.715	21.715	16.108	16.566	20.476	21.598	BN12	C9	O
24.799	34.246	35.089	34.036	21.854	21.854	16.585	22.045	18.752	19.237	23.257	23.017	21.917	25.634	25.626	20.511	20.507	20.753	20.769	20.767	20.770	20.861	18.895	21.821	21.765	21.764	21.764	15.958	16.496	20.486	21.576	Fern	C9	P
24.800	34.247	35.090	34.039	21.855	21.855	16.585	22.045	18.752	19.238	23.257	23.018	21.917	25.634	25.627	20.511	20.507	20.753	20.769	20.767	20.770	20.861	18.895	21.819	21.763	21.763	21.763	15.956	16.494	20.484	21.574	Willow	C9	P
23.364	32.630	33.971	32.384	20.818	20.818	15.745	21.013	17.818	18.337	22.423	22.491	20.719	24.440	24.433	20.555	20.550	20.810	20.811	20.810	20.813	20.904	18.949	21.148	21.095	21.095	21.095	15.399	15.859	19.916	20.971	Kawika	C9	Q
24.676	33.802	34.593	33.652	21.455	21.455	16.551	21.586	18.640	18.982	23.046	22.906	21.823	25.333	25.325	20.447	20.443	20.673	20.702	20.700	20.703	20.800	18.833	21.454	21.402	21.402	21.402	15.623	16.158	20.178	21.266	Lucielle	C9	R
24.754	34.084	34.903	33.937	21.577	21.577	16.597	21.705	18.690	19.092	23.119	22.922	21.837	25.425	25.418	20.478	20.473	20.704	20.746	20.745	20.748	20.845	18.870	21.553	21.500	21.500	21.500	15.706	16.246	20.245	21.380	Saudage	C9	R
23.946	33.302	33.994	33.035	21.158	21.158	15.959	21.460	18.034	18.611	22.749	21.742	21.164	24.861	24.853	20.502	20.498	20.728	20.753	20.751	20.754	20.839	18.858	21.605	21.557	21.557	21.557	15.942	16.438	20.326	21.414	Honeybear	C9	S
24.604	34.215	35.019	33.968	21.686	21.686	16.366	21.985	18.500	19.059	23.255	22.436	21.729	25.529	25.521	20.493	20.488	20.719	20.743	20.742	20.745	20.829	18.855	22.000	21.949	21.949	21.949	16.286	16.782	20.695	21.788	Toothless	C9	S
23.934	33.310	34.021	33.035	21.147	21.147	15.946	21.419	18.001	18.573	22.728	21.725	21.152	24.857	24.850	20.528	20.524	20.755	20.764	20.763	20.766	20.857	18.893	21.614	21.566	21.565	21.565	15.928	16.433	20.336	21.422	Pagassa	C9	S
25.008	34.636	35.499	34.399	21.921	21.921	16.619	22.244	18.846	19.307	23.546	22.983	22.169	25.920	25.912	20.484	20.479	20.713	20.734	20.732	20.735	20.819	18.842	22.086	22.034	22.034	22.034	16.281	16.799	20.730	21.841	Tadhana	C9	S
24.330	33.999	34.868	33.580	20.187	20.187	16.423	20.476	18.314	18.607	22.926	22.698	21.932	23.724	23.718	19.829	19.825	20.047	20.563	20.561	20.564	20.649	18.300	20.378	20.336	20.335	20.335	14.407	14.828	18.866	20.057	Diva	C9	T
23.189	33.271	35.412	32.664	20.177	20.177	15.548	19.874	17.191	17.868	22.724	22.084	20.628	23.051	23.046	19.843	19.839	20.061	20.572	20.570	20.573	20.658	18.292	21.607	21.558	21.557	21.557	15.578	15.963	19.828	21.033	Shelly	C9	U
23.201	33.239	35.369	32.622	20.176	20.176	15.547	19.889	17.206	17.879	22.731	22.106	20.645	23.052	23.047	19.808	19.804	20.025	20.559	20.557	20.560	20.645	18.272	21.602	21.555	21.555	21.555	15.574	15.958	19.830	21.040	Xenia	C9	U
23.843	33.341	34.106	33.139	21.374	21.374	16.355	22.011	18.502	18.517	22.328	21.822	21.176	24.803	24.798	20.074	20.069	20.319	20.706	20.705	20.708	20.800	18.192	21.738	21.686	21.686	21.686	16.574	16.939	19.863	21.372	Eltigre	C9	V
24.070	33.116	33.880	32.809	20.361	20.361	16.695	21.532	19.299	18.306	22.708	22.684	21.882	24.385	24.379	20.056	20.052	20.300	20.710	20.708	20.712	20.803	18.194	20.355	20.307	20.306	20.306	14.859	15.206	18.474	20.074	HB10c2	C9	W
24.319	33.964	34.807	33.800	21.590	21.590	16.680	22.234	18.792	18.679	22.934	22.353	21.639	25.162	25.157	20.057	20.053	20.302	20.679	20.678	20.681	20.773	18.191	21.980	21.928	21.928	21.928	16.849	17.230	20.065	21.655	Kiel007	C9	W
24.309	33.960	34.803	33.796	21.580	21.580	16.669	22.224	18.781	18.668	22.925	22.341	21.629	25.152	25.146	20.048	20.044	20.292	20.673	20.671	20.675	20.766	18.182	21.977	21.925	21.925	21.925	16.847	17.228	20.058	21.650	Redbud	C9	W
24.317	33.940	34.788	33.791	21.594	21.594	16.675	22.233	18.791	18.682	22.920	22.354	21.639	25.162	25.157	20.068	20.063	20.312	20.700	20.698	20.702	20.793	18.189	21.977	21.922	21.922	21.922	16.853	17.222	20.067	21.644	Rani	C9	W
22.843	32.703	34.513	32.589	20.226	20.226	14.972	20.791	17.057	17.944	22.201	20.886	19.804	23.731	23.726	20.186	20.182	20.409	20.941	20.939	20.942	21.028	18.847	21.634	21.585	21.584	21.584	16.144	16.500	20.048	21.432	Sitara	C9	X
23.173	33.394	35.515	32.976	20.767	20.767	15.328	20.861	17.433	18.235	23.017	21.698	20.344	24.063	24.058	19.913	19.909	20.137	20.873	20.871	20.874	20.963	18.525	22.278	22.225	22.224	22.224	16.501	16.835	20.492	21.999	Leyra	C9	Y
23.033	32.118	34.106	31.465	20.098	20.098	15.661	19.740	17.216	17.502	22.508	22.841	20.683	23.173	23.168	20.413	20.409	20.668	20.367	20.365	20.368	20.461	18.549	21.188	21.141	21.140	21.140	15.059	15.445	19.692	20.645	PBL1c	C9	Z
23.811	33.696	35.952	33.209	21.093	21.093	16.097	21.024	17.986	18.476	23.427	22.844	21.207	24.700	24.693	20.047	20.043	20.269	20.674	20.673	20.676	20.753	18.317	22.124	22.068	22.068	22.068	16.228	16.526	20.451	21.807	Likha	C9	AA
22.416	25.583	26.791	27.561	21.661	21.658	22.871	22.910	23.421	23.201	19.070	20.698	19.914	19.938	19.934	19.781	19.777	19.925	20.094	20.090	19.665	20.041	19.333	14.380	12.730	12.730	12.730	7.766	7.559	11.972	13.967	H6_R3	C10	AB
21.854	26.106	26.619	25.983	21.384	21.382	16.129	21.337	17.867	19.792	21.529	19.479	19.091	22.902	22.894	21.856	21.836	22.125	22.961	22.956	22.944	23.007	21.529	20.648	20.626	20.625	20.625	14.099	14.567	19.487	20.710	Harrison	C10	AC
21.910	26.202	26.723	26.039	21.427	21.425	16.151	21.385	17.889	19.821	21.532	19.504	19.110	22.969	22.961	21.857	21.838	22.126	22.962	22.958	22.946	23.008	21.531	20.646	20.624	20.623	20.623	14.093	14.554	19.477	20.700	Paisley	C10	AC
27.205	18.062	18.283	17.959	15.619	15.617	12.141	16.438	13.822	13.455	17.136	30.220	27.156	18.160	18.160	13.596	13.594	13.692	13.377	13.382	13.130	14.758	12.106	19.775	19.739	19.738	19.738	13.675	14.339	18.894	19.229	Lily	S10	
20.968	25.558	26.402	25.001	20.255	20.253	15.623	20.524	17.341	18.174	21.784	19.927	18.533	22.823	22.819	22.591	22.579	22.869	23.047	23.043	23.033	23.097	20.682	21.047	21.003	21.002	21.002	14.921	15.216	19.109	20.726	Diane	C11	AD
20.968	25.562	26.406	25.003	20.257	20.255	15.624	20.526	17.343	18.176	21.786	19.927	18.533	22.825	22.820	22.595	22.582	22.872	23.051	23.046	23.036	23.101	20.685	21.049	21.004	21.004	21.004	14.921	15.216	19.111	20.727	Vadim	C11	AD
20.970	25.558	26.406	25.005	20.262	20.261	15.628	20.532	17.347	18.183	21.794	19.930	18.535	22.835	22.830	22.607	22.595	22.885	23.051	23.046	23.036	23.101	20.692	21.050	21.003	21.002	21.002	14.923	15.218	19.119	20.732	Vegas	C11	AD
21.087	25.502	26.338	24.903	20.483	20.481	15.973	20.552	17.614	18.291	21.967	20.425	18.906	22.895	22.890	22.098	22.086	22.372	22.648	22.643	22.633	22.697	20.136	20.833	20.786	20.786	20.786	14.811	15.095	18.985	20.658	Hayley	C11	AD
31.716	29.440	29.431	30.490	23.243	23.243	23.338	23.904	23.408	23.407	23.651	24.945	24.998	26.167	26.167	21.106	21.113	21.241	21.134	21.131	20.613	20.696	19.949	18.898	14.543	14.543	14.543	8.278	7.691	12.686	16.243	H3_R14	C11	AD
30.627	29.484	29.486	30.569	23.098	23.100	23.009	23.880	23.083	23.117	23.433	24.159	24.175	33.870	33.871	21.202	21.220	21.379	21.135	21.132	20.560	20.818	19.998	19.741	15.280	15.280	15.280	8.617	8.179	13.247	16.703	H10_R4	C11	AE
13.202	17.685	17.592	18.251	15.164	15.165	12.090	16.332	13.804	13.514	17.580	14.083	13.950	18.103	18.100	12.231	12.234	12.302	12.086	12.085	11.896	13.298	10.974	19.156	19.009	19.009	19.009	13.108	13.806	17.882	17.928	Dragolir	C12	AF
12.498	17.167	17.052	17.463	15.219	15.221	12.253	15.508	13.378	12.990	17.030	13.804	13.651	17.346	17.343	11.361	11.348	11.415	11.046	11.045	10.868	12.184	9.687	18.739	18.595	18.595	18.595	12.981	13.670	17.407	17.501	LincolnB	C12	AG
12.637	17.205	17.094	17.504	15.256	15.257	12.294	15.542	13.454	13.010	17.050	13.835	13.678	17.334	17.331	11.366	11.353	11.427	11.051	11.050	10.873	12.190	9.697	18.743	18.599	18.599	18.599	12.985	13.667	17.417	17.512	Wanderer	C12	AG
	20.915	22.743	22.068	18.230	18.233	18.343	18.501	18.411	19.028	18.900	43.196	42.030	19.814	19.811	20.618	20.632	20.781	20.464	20.461	19.913	19.791	19.202	15.076	12.186	12.186	12.186	7.835	7.331	10.698	13.740	H3_R11	S11	
20.915		99.981	81.047	21.859	21.859	22.664	22.202	21.121	22.322	25.735	22.356	23.205	21.246	21.243	19.213	19.224	19.362	19.454	19.450	18.848	19.354	18.083	17.370	14.484	14.484	14.484	10.517	10.354	13.172	15.353	H7_R10	C13	AH
22.743	99.981		84.432	24.251	24.251	25.599	24.386	23.407	24.602	26.633	23.949	23.805	21.726	21.722	19.224	19.235	19.373	19.465	19.461	18.858	19.365	18.094	17.725	14.551	14.551	14.551	10.248	10.077	13.104	15.397	H12_R3	C13	AH
22.068	81.047	84.432		24.541	24.540	26.272	24.938	24.019	25.026	25.155	24.319	23.449	22.786	22.781	19.829	19.841	20.002	20.027	20.022	19.696	19.948	18.579	16.993	15.258	15.257	15.257	10.929	10.669	13.712	15.868	H11_R3	C13	AI
18.230	21.859	24.251	24.541		99.993	57.234	58.652	53.397	50.800	42.665	21.858	21.166	20.904	20.905	19.118	19.119	19.248	18.716	18.716	18.370	18.891	17.648	15.698	13.286	13.286	13.286	9.048	8.908	12.862	15.086	H3_R15	C14	AJ
18.233	21.859	24.251	24.540	99.993		57.234	58.649	53.391	50.800	42.665	21.860	21.168	20.910	20.911	19.117	19.117	19.247	18.711	18.712	18.366	18.887	17.647	15.699	13.288	13.287	13.287	9.049	8.910	12.863	15.088	H10_R5	C14	AJ
18.343	22.664	25.599	26.272	57.234	57.234		53.711	58.682	50.127	42.477	23.421	22.498	20.752	20.752	18.847	18.844	19.032	18.412	18.413	18.036	18.547	17.159	12.688	11.256	11.256	11.256	6.015	5.849	10.061	12.318	H14_R10	S12	
18.501	22.202	24.386	24.938	58.652	58.649	53.711		71.379	54.221	44.168	22.499	21.830	24.971	24.971	19.524	19.529	19.706	19.261	19.260	18.926	19.232	18.169	16.477	14.617	14.617	14.617	9.968	9.774	13.318	15.768	H14_R7	C15	AK
18.411	21.121	23.407	24.019	53.397	53.391	58.682	71.379		50.778	41.112	23.196	22.466	19.623	19.622	18.846	18.851	19.030	18.491	18.489	18.212	18.415	17.118	13.677	12.235	12.234	12.234	7.055	6.922	10.775	13.198	H14_R8	C15	AL
19.028	22.322	24.602	25.026	50.800	50.800	50.127	54.221	50.778		61.374	21.740	20.896	19.864	19.866	19.702	19.697	19.863	19.659	19.659	19.383	19.117	18.546	14.542	12.653	12.653	12.653	7.892	7.594	11.752	14.118	H9_R14	C16	AM
18.900	25.735	26.633	25.155	42.665	42.665	42.477	44.168	41.112	61.374		22.171	20.231	21.492	21.495	19.684	19.678	19.871	19.781	19.785	19.532	18.608	18.433	18.029	17.321	17.321	17.321	10.755	10.366	14.959	17.158	H9_R15	C16	AN
43.196	22.356	23.949	24.319	21.858	21.860	23.421	22.499	23.196	21.740	22.171		100.000	19.783	19.777	19.688	19.708	19.871	19.273	19.278	18.815	18.596	17.752	15.694	13.933	13.933	13.933	7.030	6.750	11.649	14.780	H14_R3	C17	AO
42.030	23.205	23.805	23.449	21.166	21.168	22.498	21.830	22.466	20.896	20.231	100.000		19.690	19.683	19.762	19.782	19.955	19.338	19.343	19.100	18.631	17.749	14.614	13.562	13.562	13.562	7.535	7.318	11.603	14.369	phiERICV	C17	AO
19.814	21.246	21.726	22.786	20.904	20.910	20.752	24.971	19.623	19.864	21.492	19.783	19.690		99.956	17.540	17.553	17.695	17.062	17.059	16.790	16.913	16.527	15.683	14.314	14.315	14.315	11.001	10.686	13.203	15.427	H3_R18	C18	AP
19.811	21.243	21.722	22.781	20.905	20.911	20.752	24.971	19.622	19.866	21.495	19.777	19.683	99.956		17.539	17.552	17.694	17.061	17.057	16.789	16.911	16.526	15.681	14.312	14.314	14.314	11.002	10.688	13.201	15.426	H6_R1	C18	AP
20.618	19.213	19.224	19.829	19.118	19.117	18.847	19.524	18.846	19.702	19.684	19.688	19.762	17.540	17.539		99.832	96.191	75.823	75.810	74.620	73.704	72.604	63.028	48.660	48.660	48.660	13.542	13.299	35.250	46.129	Ash	C19	AQ
20.632	19.224	19.235	19.841	19.119	19.117	18.844	19.529	18.851	19.697	19.678	19.708	19.782	17.553	17.552	99.832		96.177	75.812	75.799	74.609	73.693	72.655	63.053	48.678	48.678	48.678	13.538	13.299	35.233	46.113	Ley	C19	AQ
20.781	19.362	19.373	20.002	19.248	19.247	19.032	19.706	19.030	19.863	19.871	19.871	19.955	17.695	17.694	96.191	96.177		74.040	74.027	72.860	71.875	75.297	61.293	47.376	47.376	47.376	13.718	13.485	35.878	46.860	C7Cdelta	C19	AQ
20.464	19.454	19.465	20.027	18.716	18.711	18.412	19.261	18.491	19.659	19.781	19.273	19.338	17.062	17.061	75.823	75.812	74.040		99.966	94.120	87.889	63.260	75.404	57.339	57.339	57.339	12.455	12.316	33.002	43.027	Halcyone	C19	AR
20.461	19.450	19.461	20.022	18.716	18.712	18.413	19.260	18.489	19.659	19.785	19.278	19.343	17.059	17.057	75.810	75.799	74.027	99.966		94.087	87.871	63.248	75.410	57.343	57.343	57.343	12.446	12.308	32.995	43.020	Heath	C19	AR
19.913	18.848	18.858	19.696	18.370	18.366	18.036	18.926	18.212	19.383	19.532	18.815	19.100	16.790	16.789	74.620	74.609	72.860	94.120	94.087		84.131	62.665	73.617	56.939	56.939	56.939	12.328	12.188	32.612	42.519	Scottie	C19	AR
19.791	19.354	19.365	19.948	18.891	18.887	18.547	19.232	18.415	19.117	18.608	18.596	18.631	16.913	16.911	73.704	73.693	71.875	87.889	87.871	84.131		60.788	76.007	56.804	56.804	56.804	12.900	13.327	34.646	44.794	Unity	C19	AS
19.202	18.083	18.094	18.579	17.648	17.647	17.159	18.169	17.118	18.546	18.433	17.752	17.749	16.527	16.526	72.604	72.655	75.297	63.260	63.248	62.665	60.788		47.147	37.207	37.207	37.207	15.472	14.987	41.540	43.439	Tripp	C19	AT
15.076	17.370	17.725	16.993	15.698	15.699	12.688	16.477	13.677	14.542	18.029	15.694	14.614	15.683	15.681	63.028	63.053	61.293	75.404	75.410	73.617	76.007	47.147		99.925	99.927	99.927	35.727	36.529	56.816	63.730	H7_R1	C20	AU
12.186	14.484	14.551	15.258	13.286	13.288	11.256	14.617	12.235	12.653	17.321	13.933	13.562	14.314	14.312	48.660	48.678	47.376	57.339	57.343	56.939	56.804	37.207	99.925		99.998	99.998	33.665	34.726	54.704	59.599	H8_R12	C20	AU
12.186	14.484	14.551	15.257	13.286	13.287	11.256	14.617	12.234	12.653	17.321	13.933	13.562	14.315	14.314	48.660	48.678	47.376	57.339	57.343	56.939	56.804	37.207	99.927	99.998		100.000	33.666	34.728	54.706	59.601	H11_R8	C20	AU
12.186	14.484	14.551	15.257	13.286	13.287	11.256	14.617	12.234	12.653	17.321	13.933	13.562	14.315	14.314	48.660	48.678	47.376	57.339	57.343	56.939	56.804	37.207	99.927	99.998	100.000		33.666	34.728	54.706	59.601	H12_R8	C20	AU
7.835	10.517	10.248	10.929	9.048	9.049	6.015	9.968	7.055	7.892	10.755	7.030	7.535	11.001	11.002	13.542	13.538	13.718	12.455	12.446	12.328	12.900	15.472	35.727	33.665	33.666	33.666		78.742	40.662	39.090	H9_R10	C21	AV
7.331	10.354	10.077	10.669	8.908	8.910	5.849	9.774	6.922	7.594	10.366	6.750	7.318	10.686	10.688	13.299	13.299	13.485	12.316	12.308	12.188	13.327	14.987	36.529	34.726	34.728	34.728	78.742		42.695	39.771	H14_R2	C21	AW
10.698	13.172	13.104	13.712	12.862	12.863	10.061	13.318	10.775	11.752	14.959	11.649	11.603	13.203	13.201	35.250	35.233	35.878	33.002	32.995	32.612	34.646	41.540	56.816	54.704	54.706	54.706	40.662	42.695		65.154	H10_R7	C22	AX
13.740	15.353	15.397	15.868	15.086	15.088	12.318	15.768	13.198	14.118	17.158	14.780	14.369	15.427	15.426	46.129	46.113	46.860	43.027	43.020	42.519	44.794	43.439	63.730	59.599	59.601	59.601	39.090	39.771	65.154		H14_R9	C22	AY

6.4 Discussion

Temperate phages can remain in a dormant state within their host without triggering the lytic cycle, while at the same time having a considerable impact on the host genome variability and evolution, modulating the host fitness and virulence (Brussow et al., 2004; Harrison & Brockhurst, 2017). To our knowledge, there has been no attempts to explore the role of temperate phages in the ecology and evolution of *P. larvae* despite studies reporting a total of 51 *P. larvae* prophages in the last eight years (Abraham et al., 2016; Beims et al., 2020, 2015; Bozdeveci et al., 2021; Carson et al., 2015; Bryan D Merrill et al., 2018; A. Oliveira et al., 2013; Ribeiro et al., 2019; Tsourkas et al., 2015; Walker et al., 2018; Yost et al., 2018).

Here, 11 complete genomes of *P. larvae* previously isolated from AFB outbreaks (Alippi, Lopez, & Aguilar, 2002; Beims et al., 2020; Djukic et al., 2014; Genersch et al., 2005; Heyndrickx et al., 1996; Rosa et al., 2015) were analysed to identify the presence of intact prophages. In total, 55 full-length *P. larvae* phage genomes were identified and analysed *in silico* and their potential to influence forthcoming generations by providing new features was investigated.

Prophages were identified in the *P. larvae* genomes using both the software PHASTER and by manual curing. The manual curing indicated an incorrect estimate of prophages (both intact and defective) in the software analyses (**Table 19**). Despite PHASTER is a commonly used software for prophage prediction, some inaccuracies, are being described (Arndt, Marcu, Liang, & Wishart, 2019).

Surprisingly, the set of *P. larvae* plasmids identified did not hold any intact prophage, contrarily to what has been reported for other species, such as *Acinetobacter baumannii* (Costa et al., 2018). Only defective prophages were found in the *P. larvae* plasmids. A similar phenomenon was observed in *Clostridium difficile* DLL3026 plasmids, with some defective prophages encoding structural and integrase genes (Amy et al., 2018).

There was no apparent association between ERIC genotype and the number of prophages per genome. On average, each *P. larvae* strain harboured five intact prophages. The presence of multiple intact prophages in a single strain (poly-lysogenic strains) has been reported for both Gram-positive and Gram-negative bacteria (Garriss & Henriques-Normark, 2020; Touchon, Bernheim, & Rocha, 2016). In Gram-positive bacteria, up to five prophages have been observed in a single *C. difficile* genome (L. Fortier, 2018), around two prophages per genome have been reported for *Bacillus thuringiensis* (Fu, Wu, Yuan, & Gao, 2019) and *Lactococcus lactis* (Ruiz-cruz et al., 2020), and in *S. aureus* four prophages have been

observed (Bae, Baba, Hiramatsu, & Schneewind, 2006). In Gram-negative bacteria, more than two prophages per genome have been reported for *Citrobacter rodentium* and *Klebsiella pneumoniae* (Bleriot et al., 2020; Magaziner, Zeng, Chen, & Salmond, 2019) and for enterotoxigenic *E. coli* more than eight prophages per genome (Wang et al., 2020). Because of the superinfection exclusion mechanism that prevents infection by similar phages, a poly-lysogenic strain may become less susceptible to accepting new prophages (Touchon et al., 2016).

As expected, the presented data suggest a positive correlation between the number of integrated prophages and the size of the genome of bacteria. If on the one hand, the presence of so many intact prophages increases the length of the host genome, on the other hand, larger genomes provide higher stability to hold prophages (Costa et al., 2018; Touchon et al., 2016). From an evolutionary point of view, it is possible that hosts harbouring more prophages do not benefit from the integration of new prophages and consequently will not accept genes providing novel advantageous functions.

The comparison between the whole genomes of the newly identified and the previously described *P. larvae* phages (Abraham et al., 2016; Beims et al., 2020, 2015; Carson et al., 2015; Bryan D Merrill et al., 2018; A. Oliveira et al., 2013; Ribeiro et al., 2019; Tsourkas et al., 2015; Walker et al., 2018; Yost et al., 2018) proposes changes in some of the existent clusters (Vegas and Harrison (Ribeiro et al., 2019)) and introduces new ones (more 18 clusters and 10 singletons). Before this study, four clusters (Fern, Harrison, Vegas, Halcyone) and two singletons, (API480 and Lily) had been reported (Ribeiro et al., 2019) and recently, Bozdeveci et al., 2021 introduced a new phage similar to API480, proposing a new cluster. This new data brings new insights into the diversity of *P. larvae* phages and supports the importance of a permanent feed and update of the available phage genomic data.

Besides genomics, the present analysis also contributes to widening the taxonomic diversity of *P. larvae* phages. So far, most of the reported phages are siphoviruses (Beims et al., 2020; A. Oliveira et al., 2013; Stamereilers et al., 2018), with the exception of two podoviruses (Bozdeveci et al., 2021; Ribeiro et al., 2019). The present analysis suggests the inclusion of at least 15 myoviruses in the collection. Furthermore, this suggests a discussion on the classification of phage Lily and homologous as myoviruses. Concerning Lily phage, it is of note that, besides being reported as a member of the *Siphoviridae* family, Lilly encodes proteins typically found in myoviruses such as TSP, TMP and one TAP (gp15), the tail tube protein (gp14), tail fiber proteins (Stamereilers et al., 2018) and lacks the MTP gene. This might introduce some ambiguity in comparisons with such phage.

Our prophage analysis further disclosed a set of proteins that can putatively influence host fitness and pathogenesis. Despite the lower incidence of CDS originating these categories (**Figure 40**), they seem to be the most impacting on the host phenotypic transformation. For example, the small multidrug resistance (SMR) transporter, found in three of the analysed prophages, seems to provide the cell the ability to escape antibiotics by transporting drugs out of the cell. Partial sequences of AMR (e.g., genes encoding inhibitory proteins of the β-lactam antibiotics class A and B) or CDS with regulatory functions concerning the tetracycline resistance mechanism were apparently observed, but contrarily what has already been reported for other bacteria (Stanczak-Mrozek, Laing, & Lindsay, 2017; Wachino, Jin, Kimura, & Arakawa, 2019), none of the analysed prophages are able to provide the host a functional AMR. They might be a result of previous attempts to use antibiotics to control AFB in hives (Nakajima, Okayama, Sakogawa, Nakamura, & Hayama, 1997; Reybroeck, Daeseleire, De Brabander, & Herman, 2012).

Our analysis also suggests the presence of proteins involved in the transport of either generic (ATP-binding cassette (ABC transporter), MFS transporter, efflux transporter) or specific (aromatic acid exporter, Fe-S cluster transporter) substances. The bioavailability of iron has been shown to be recognisably vital for *P. larvae* growth (Hertlein et al., 2014). This makes the identification of proteins involved in iron uptake (SufB and NifU protein) in two prophages particularly interesting, as they may provide the host with important fitness advantages. Although the YncE protein is of unknown function it has been assumed in the past that was related to iron metabolism (McHugh et al., 2003), but a more recent characterization showed that it is associated with DNA-binding activities (Kagawa, Sagawa, Niki, & Kurumizaka, 2011).

Prophage genomes also harbour proteins with homology to others associated with *P. larvae* metabolism and regulation. Usually, the histidine kinase enzyme plays role in signal transduction across the cellular membrane by phosphotransfer and phosphatase activity. Here, a histidine kinase-like protein was identified in one prophage, and perhaps this could induce the host to phosphorylate the response regulator *agrC* associated with a common quorum sensing system, something that was previously reported in a *C. difficile* prophage (Hargreaves, Kropinski, & Clokie, 2014a, 2014b; Taylor, Fitzpatrick, Islam, & Maxwell, 2019). The presence of ComEC/Rec2, a protein enabling DNA internalization was identified in two prophages and can confer the ability for uptake of exogenous DNA from the environment, promoting the HGT (Solomon & Grossman, 1996). The enzyme phosphomannomutase was identified in several of the analysed prophages. This enzyme may play a role in several functions involving biofilm formation (biosynthesis of bacterial exopolysaccharides), protection against environmental factors and the actions of antibiotics (Regni, Tipton, & Beamer, 2002).

Although it has been reported that prophages may strongly impact bacterial virulence by providing new toxins through lysogenic conversion, as described for botulism toxin in *C. botulinum*, Shiga toxin in *E. coli* or Cholera toxin in *V. cholerae* (Barksdale & Arden, 1974; Feiner et al., 2015; O'Brien et al., 1984; Waldor & Mekalanos, 1996), the present suggests that some toxins encoded by *P. larvae* prophages may only influence the strain itself through the presence of TA systems rather than affecting bee larvae. The activity of TA systems usually leads to the inhibition of cell growth by interfering with several cellular processes. Biologically, their functions are generally associated with growth control, defence against phages, biofilm formation, persistence, programmed cell death and general stress response (Unterholzner, Poppenberger, & Rozhon, 2013; Wen, Behiels, & Devreese, 2014). In the present study, either toxin HicA or antitoxin HicB of the *hicAB* cassette were identified in several prophages. Its biological role in *P. larvae* still needs further elucidation, but the *hicAB* cassette significantly influences the mRNA translation process in other bacteria with proposed functions including persister cell formation and involvement in extra cytoplasmic stress responses (Butt et al., 2014; Li et al., 2016; Thomet, Trautwetter, Ermel, & Blanco, 2019). The presence of *hicAB* in bacterial genomes has also been associated with HGT (Butt et al., 2014; Li et al., 2016; Thomet et al., 2019). In several prophages, CDS for the antitoxin protein of a TA system were identified, namely the antitoxin part of MazEF and SocAB. MazE antitoxin is the inhibitor of MazF toxin that cleaves mRNA resulting in cellular growth arrest (Simanshu, Yamaguchi, Park, Inouye, & Patel, 2013). SocA antitoxin acts as a proteolytic adapter promoting the disruption of SocB inhibiting DNA replication (Aakre, Phung, Huang, & Laub, 2013). It can be speculated that phages harbor these antitoxins as a defence mechanism, in order to avoid host self-regulation by degradation, not as an added value to their lysogens.

In addition to TA systems, toxins that could affecting the bee larvae were also found in our analysis in prophages, which may can impact *P. larvae* virulence. The EtxB is a toxin that cause enterotoxemia in ruminants and hemolysis in human cell lines (Xin & Wang, 2019) and the sub-unit LukF-PV of the PVL toxin is responsible for the polymerization F component interspersing with S component LukS-PV to form a pore in the target host cell (Spaan, van Strijp, & Torres, 2017), both are pore-forming toxins that among other features are involved in tissue necrosis. The first, the EtxB toxin, has previously been associated with *Clostridium perfringens* (Popoff, 2011; Xin & Wang, 2019) and the latter, the PVL toxin, has been found in prophages in lysogenic *S. aureus* strains (Coombs, Baines, Howden, Swenson, & O'Brien, 2020; Diene et al., 2017). These genes might have been transferred from such bacteria to *P. larvae* by HGT, hypothesis supported by Djukic et al., (2014) and based on the observation of other toxins shares similarities in the different species (Ebeling, Fünfhaus, & Genersch, 2021).

Other prophage CDS seem to influence and increase of AFB severity because might be involved in functions with some effect on larvae tissues. A *P. larvae* infection starts with the bacteria proliferating in the larval gut before it breaches the epithelial layer and invades the haemocoel (Yue, Nordhoff, Wieler, & Genersch, 2008). The epithelial layer is lined with a peritrophic membrane consisting of chitins and glycoproteins (Konno & Mitsuhashi, 2019). The degradation of the peritrophic membrane has been shown to be a crucial part of the AFB pathogenesis as it allows direct contact with the epithelial layer and the degraded chitin may serve as a carbon source for *P. larvae* (Ebeling et al., 2016; Garcia-Gonzalez & Genersch, 2013; Garcia-Gonzalez et al., 2014). In this study, CDS for chitin-binding proteins and other proteins that may be involved in the degradation of the peritrophic membrane were identified. One of the prophages, may encode the epithelial and chitin-binding protein GbpA, previously identified in *V. cholerae,* as mediator of bacterial adhesion to human intestinal cells (Kirn, Jude, & Taylor, 2005). If we assume an analogy with bees, this might confer an advantage *P. larvae,* increase virulence of the host strain by improving bacterial colonization in the larval intestine. In another prophage, enhancin-homologous proteins, belonging to the metallopeptidase family, were observed. Originally described for viruses, enhancin is known to promote infections by degrading the peritrophic membrane of the insect gut. However, enhancin-like proteins has also been found in bacteria, including *P. larvae* (Djukic et al., 2014; Slavicek, 2012) or *Melissococcus plutonius* (Nakamura et al., 2021). In the latter, the causative agent of the honeybee brood disease European Foulbrood (EFB), it is also involved in the degradation of the peritrophic membrane (Nakamura et al., 2021). Another group of proteins from this study belong to the YopX family. Usually associated with pathogenicity, by acting as chaperones for other proteins, they also modulate host cell signalling responses through the type III secretion system (TTSS). Such proteins have also been reported for *Staphylococcus epidermidis* (Gutiérrez, Martínez, Rodríguez, & García, 2012) and *S. aureus* prophages (Diene et al., 2017), and for *Lactobacillus plantarum* virulent phages (Kyrkou et al., 2019).

The association between intact prophage CDS and a specific ERIC genotype was evaluated, even recognizing the low number of fully sequenced *P. larvae* genomes in GenBank some conjectures have been formulated regarding their connection with ERIC-genotypes. ERIC I-type strains were the less virulent strains to larvae (Rauch et al., 2009) but also with more genomes available, hold more phage-origin exclusive CDS involved in metabolism (e.g., histidine kinase-like protein, pyruvate dehydrogenase E1, dCMP deaminase family protein, efflux transporter, etc.) than virulence (enhancin and closticin (an antibacterial peptide that inhibits the growth of other bacteria) (Kemperman et al., 2003)) (**Table 22**). The higher frequency of these prophage CDS might be related to the high prevalence of this genotype in

AFB outbreaks (Tsourkas, 2020), increasing the opportunity for prophage exchange and acquisition of new genes by HGT. The analysis also revealed that CDS exclusively identified in prophages from ERIC II–V should be able to affect the fitness and virulence. ERIC II strains had proteins with the function of sporulation, membrane transporters, DNA replication and DNA mismatch repair, ERIC III through PgpA participates into the glycerophospholipid metabolism and ERIC IV had proteins related to iron uptake. Prophages from the ERIC V strain, known as a fast larvae killer (Beims et al., 2020), potentially contribute to such trait, encoding virulence genes like leukocidin subunit LukF-PV and GbpA.

Overall, despite the identification of exclusive CDS of all ERIC genotypes, it is not clear whether the CDS involved in virulence are influencing the course of the larval infection. Nevertheless, one exception that seems evident is the presence of toxins in the ERIC V strain analysed. The remaining exclusive CDS seem to be related with fitness or contributing to *P. larvae* competition with other bacteria.

Yost et al., (2016) earlier suggested that phages displayed host preference for the ERIC group from which they were isolated, and therefore, the possibility of prophages to influence a given genotype was explored, even recognizing the low number of fully sequenced *P. larvae* genomes in GenBank.

Prophages seem to be stable, specific, and important for their ERIC-genotype strains, which allows to infer that the infection of different ERIC-type strains by the same phage is unlikely. This behaviour will prevent the occurrence of HGT among the different virulent genotypes.

Within the same host, prophages usually share few similarities, as observed in *B. thuringiensis* (Fu et al., 2019). However, as expected and previously observed in staphylococci phage analyses (H. Oliveira et al., 2019), the intact prophages have high identity within the same cluster, being all morphologically similar. Furthermore, each cluster harbour intact prophages from the same ERIC or from the closest ERIC genotypes (ERIC I and II or ERIC III and IV strains (Papić, Diricks, & Kušar, 2021)). This supports the predisposition of prophages to infect *P. larvae* from the same ERIC genotype from which they were previously isolated, as previously suggested (Yost et al., 2016).

Overall, this study identified new intact prophages present in all *P. larvae* strains sequenced so far and explored their genomes concerning the potential impact on host strains. Despite some limitations of the *in silico* tools to predict and re-size prophages and the low representativeness on *P. larvae* strains diversity, we introduced important knowledge to the study of *P. larvae* phages by increasing the number of prophage genomes available and annotated.

Moreover, even if phage CDS function was not experimentally confirmed, their diversity in *P. larvae* genomes gave relevant insights on the role of prophages in such pathogen, as relevant matches were found in the database.

References

Aakre, C. D., Phung, T. N., Huang, D., & Laub, M. T. (2013). A Bacterial Toxin Inhibits DNA Replication Elongation through a Direct Interaction with the β Sliding Clamp. *Molecular Cell*, *52*(5), 617–628. https://doi.org/10.1016/j.molcel.2013.10.014

Abraham, J., Bousquet, A., Bruff, E., Carson, N., Clark, A., Connell, A., ... Miller, E. S. (2016). Paenibacillus larvae Phage Tripp Genome Has 378-Base-Pair Terminal Repeats. *Genome Announcements*, *4*(1), 1498–15. https://doi.org/10.1128/genomeA.01498-15

Aksyuk, A. A., Bowman, V. D., Kaufmann, B., Fields, C., Klose, T., Holdaway, H. A., ... Rossmann, M. G. (2012). Structural investigations of a Podoviridae streptococcus phage C1, implicationsfor the mechanism of viral entry. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(35), 14025–14026. https://doi.org/10.1073/pnas.1207730109

Alcock, B. P., Raphenya, A. R., Lau, T. T. Y., Tsang, K. K., Bouchard, M., Edalatmand, A., ... McArthur, A. G. (2020). CARD 2020: Antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Research*, *48*(D1), D517–D525. https://doi.org/10.1093/nar/gkz935

Alippi, A. ., Lopez, A. ., & Aguilar, O. . (2002). Differentiation of Paenibacillus larvae sub sp . larvae, the Cause of American Foulbrood of Honeybees, by Using PCR and Restriction Fragment Analysis of Genes Encoding 16S rRNA. *Applied and Environmental Microbiology*, *68*(7), 3655–3660. https://doi.org/10.1128/AEM.68.7.3655–3660.2002

Amy, J., Bulach, D., Knight, D., Riley, T., Johanesen, P., & Lyras, D. (2018). Identification of large cryptic plasmids in Clostridioides (Clostridium) difficile. *Plasmid*, *96–97*, 25–38. https://doi.org/10.1016/j.plasmid.2018.04.001

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., & Wishart, D. S. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Research*, *44*(W1), W16–W21. https://doi.org/10.1093/nar/gkw387

Arndt, D., Marcu, A., Liang, Y., & Wishart, D. S. (2019). PHAST , PHASTER and PHASTEST : Tools for finding prophage in bacterial genomes, *20*(July 2017), 1560–1567. https://doi.org/10.1093/bib/bbx121

Bae, T., Baba, T., Hiramatsu, K., & Schneewind, O. (2006). Prophages of Staphylococcus aureus Newman and their contribution to virulence. https://doi.org/10.1111/j.1365-2958.2006.05441.x

Barksdale, L., & Arden, S. B. (1974). Persisting Bacteriophage Infections, Lysogeny, and Phage Conversions. *Annual Review of Microbiology*, *28*(1), 265–300. https://doi.org/10.1146/annurev.mi.28.100174.001405

Beims, H., Bunk, B., Erler, S., Mohr, K. I., Spröer, C., Pradella, S., ... Steinert, M. (2020). Discovery of Paenibacillus larvae ERIC V: Phenotypic and genomic comparison to genotypes ERIC I-IV reveal different inventories of virulence factors which correlate with epidemiological prevalences of American Foulbrood. *International Journal of Medical Microbiology*, *310*(2), 151394. https://doi.org/10.1016/j.ijmm.2020.151394

Beims, H., Wittmann, J., Bunk, B., Spröer, C., Rohde, C., Günther, G., ... Steinert, M. (2015). Paenibacillus larvae-Directed bacteriophage HB10c2 and its application in American foulbrood-affected honey bee larvae. *Applied and Environmental Microbiology*, *81*(16), 5411–5419. https://doi.org/10.1128/AEM.00804-15

Bensing, B. A., Siboo, I. R., & Sullam, P. M. (2001). Proteins PblA and PblB of Streptococcus mitis , Which Promote Binding to Human Platelets, Are Encoded within a Lysogenic Bacteriophage. *Infection and Immunity*, *69*(10), 6186–6192. https://doi.org/10.1128/IAI.69.10.6186-6192.2001

Bleriot, I., Trastoy, R., Blasco, L., Cuenca, F. F.-, Ambroa, A., Fernández-, L., ... Navarro, F. (2020). Genomic analysis of 40

prophages located in the genomes of 16 carbapenemase-producing clinical strains of Klebsiella pneumoniae. *Microbial Genomics*. https://doi.org/10.1099/mgen.0.000369

Bobay, L.-M., Touchon, M., & Rocha, E. P. C. (2014). Pervasive domestication of defective prophages by bacteria. *Proceedings of the National Academy of Sciences*, *111*(33), 12127–12132. https://doi.org/10.1073/pnas.1405336111

Bondy-Denomy, J., & Davidson, A. R. (2014). When a virus is not a parasite: The beneficial effects of prophages on bacterial fitness. *Journal of Microbiology*, *52*(3), 235–242. https://doi.org/10.1007/s12275-014-4083-3

Bozdeveci, A., Akpınar, R., & Karaoğlu, Ş. A. (2021). Isolation, characterization, and comparative genomic analysis of vB_PlaP_SV21, new bacteriophage of Paenibacillus larvae. *Virus Research*, *305*(September), 198571. https://doi.org/10.1016/j.virusres.2021.198571

Brussow, H., Canchaya, C., & Hardt, W. (2004). Phages and the Evolution of Bacterial Pathogens: from Genomic Rearrangements to Lysogenic Conversion. *Microbiology and Molecular Biology Reviews*, *68*(3), 560–602. https://doi.org/10.1128/MMBR.68.3.560

Butt, A., Higman, V. A., Williams, C., Crump, M. P., Hemsley, C. M., Harmer, N., & Titball, R. W. (2014). The HicA toxin from Burkholderia pseudomallei has a role in persister cell formation. *Biochemical Journal*, *459*(2), 333–344. https://doi.org/10.1042/BJ20140073

Carson, S., Bruff, E., DeFoor, W., Dums, J., Groth, A., Hatfield, T., … Miller, E. S. (2015). Genome Sequences of Six Paenibacillus larvae Siphoviridae Phages. *Genome Announcements*, *3*(3), e00101-15. https://doi.org/10.1128/genomeA.00101-15

Casjens, S. (2003). Prophages and bacterial genomics: What have we learned so far? *Molecular Microbiology*, *49*(2), 277–300. https://doi.org/10.1046/j.1365-2958.2003.03580.x

Coombs, G. W., Baines, S. L., Howden, B. P., Swenson, K. M., & O'Brien, F. G. (2020). Diversity of bacteriophages encoding Panton-Valentine leukocidin in temporally and geographically related Staphylococcus aureus. *PLoS ONE*, *15*(2), 1–17. https://doi.org/10.1371/journal.pone.0228676

Costa, A. R., Monteiro, R., & Azeredo, J. (2018). Genomic analysis of *Acinetobacter baumannii* prophages reveals remarkable diversity and suggests profound impact on bacterial virulence and fitness. *Cold Spring Harbor Laboratory Press*, *March 23*(May), 1–11. https://doi.org/https://doi.org/10.1101/286476

Czajkowski, R. (2019). May the phage be with you? Prophage-like elements in the genomes of soft rot Pectobacteriaceae: Pectobacterium spp. and Dickeya spp. *Frontiers in Microbiology*, *10*(FEB). https://doi.org/10.3389/fmicb.2019.00138

Diene, S. M., Corvaglia, A. R., François, P., van der Mee-Marquet, N., Amirault, P., Lehiani, O., … Petit le Gouas, G. (2017). Prophages and adaptation of Staphylococcus aureus ST398 to the human clinic. *BMC Genomics*, *18*(1), 1–10. https://doi.org/10.1186/s12864-017-3516-x

Dingman, D. W., Bakhiet, N., Field, C. C., & Stahly, D. P. (1984). Isolation of two bacteriophages from Bacillus larvae, PBL1 and PBL0.5, and partial characterization of PBL1. *Journal of General Virology*, *65*, 1101–1105.

Djukic, M., Brzuszkiewicz, E., Fünfhaus, A., Voss, J., Gollnow, K., Poppinga, L., … Daniel, R. (2014). How to kill the honey bee larva: Genomic potential and virulence mechanisms of Paenibacillus larvae. *PLoS ONE*, *9*(3), e90914. https://doi.org/10.1371/journal.pone.0090914

Ebeling, J., Fünfhaus, A., & Genersch, E. (2021). The Buzz about ADP-Ribosylation Toxins from Paenibacillus larvae, the Causative Agent of American Foulbrood in Honey Bees. *Toxins*, *13*(2), 151. https://doi.org/10.3390/toxins13020151

Ebeling, J., Knispel, H., Hertlein, G., Fünfhaus, A., & Genersch, E. (2016). Biology of Paenibacillus larvae, a deadly pathogen of honey bee larvae. *Applied Microbiology and Biotechnology*, *100*(17), 7387–7395. https://doi.org/10.1007/s00253-016-7716-0

European Parliament and the Council of the European Union. (2010). Commission regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuff of animal origin. *Official Journal of the European Union*, (L 15/1).

Feiner, R., Argov, T., Rabinovich, L., Sigal, N., Borovok, I., & Herskovits, A. A. (2015). A new perspective on lysogeny: prophages as active regulatory switches of bacteria. *Nature Reviews Microbiology*, *13*(10), 641–650. https://doi.org/10.1038/nrmicro3527

Figueroa-Bossi, N., Uzzau, S., Maloriol, D., & Bossi, L. (2001). Variable assortment of prophages provides a transferable repertoire of pathogenic determinants in Salmonella. *Molecular Microbiology*, *39*(2), 260–272. https://doi.org/10.1046/j.1365-2958.2001.02234.x

Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., ... Punta, M. (2014). Pfam: The protein families database. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkt1223

Fortier, L. (2017). The Contribution of Bacteriophages to the Biology and Virulence of Pathogenic Clostridia. In S. Sariaslani & G. M. Gadd (Eds.), *Advances in Applied Microbiology* (Vol. 101, pp. 169–200). Elsevier Ltd. https://doi.org/10.1016/bs.aambs.2017.05.002

Fortier, L. (2018). Bacteriophages Contribute to Shaping Clostridioides (Clostridium) difficile Species. *Frontiers in Microbiology*, *9*(August), 1–10. https://doi.org/10.3389/fmicb.2018.02033

Fortier, L. C., & Sekulovic, O. (2013). Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence*, *4*(5), 354–365. https://doi.org/10.4161/viru.24498

Fu, Y., Wu, Y., Yuan, Y., & Gao, M. (2019). Prevalence and Diversity Analysis of Candidate Prophages to Provide An Understanding on Their Roles in Bacillus Thuringiensis. *Viruses*.

Garcia-Gonzalez, E., & Genersch, E. (2013). Honey bee larval peritrophic matrix degradation during infection with Paenibacillus larvae, the aetiological agent of American foulbrood of honey bees, is a key step in pathogenesis. *Environmental Microbiology*, *15*(11), 2894–2901. https://doi.org/10.1111/1462-2920.12167

Garcia-Gonzalez, E., Poppinga, L., Fünfhaus, A., Hertlein, G., Hedtke, K., Jakubowska, A., & Genersch, E. (2014). Paenibacillus larvae Chitin-Degrading Protein PlCBP49 Is a Key Virulence Factor in American Foulbrood of Honey Bees. *PLoS Pathogens*, *10*(7), e1004284. https://doi.org/10.1371/journal.ppat.1004284

Garriss, G., & Henriques-Normark, B. (2020). Lysogeny in Streptococcus pneumoniae. *Microorganisms*, *8*(10), 1546. https://doi.org/10.3390/microorganisms8101546

Genersch, E. (2010). American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. *Journal of Invertebrate Pathology*, *103*(SUPPL. 1), S10–S19. https://doi.org/10.1016/j.jip.2009.06.015

Genersch, E. (2017). Beekeeping – From Science to Practice, 157–174. https://doi.org/10.1007/978-3-319-60637-8

Genersch, E., Ashiralieva, A., & Fries, I. (2005). Strain- and Genotype-Specific Differences in Virulence of Paenibacillus larvae subsp. larvae, a Bacterial Pathogen Causing American Foulbrood Disease in Honeybees. *Applied and Environmental Microbiology*, *71*(11), 7551–7555. https://doi.org/10.1128/AEM.71.11.7551-7555.2005

Gutiérrez, D., Martínez, B., Rodríguez, A., & García, P. (2012). Genomic characterization of two Staphylococcus epidermidis bacteriophages with anti-biofilm potential. *BMC Genomics*, *13*(1). https://doi.org/10.1186/1471-2164-13-228

Hargreaves, K. R., Kropinski, A. M., & Clokie, M. R. (2014a). Bacteriophage behavioral ecology. *Bacteriophage*, *4*(3), e29866. https://doi.org/10.4161/bact.29866

Hargreaves, K. R., Kropinski, A. M., & Clokie, M. R. J. (2014b). What does the talking? Quorum sensing signalling genes discovered in a bacteriophage genome. *PLoS ONE*, *9*(1). https://doi.org/10.1371/journal.pone.0085131

Harrison, E., & Brockhurst, M. A. (2017). Ecological and Evolutionary Benefits of Temperate Phage: What Does or Doesn't Kill You Makes You Stronger. *BioEssays*, *39*(12). https://doi.org/10.1002/bies.201700112

Hertlein, G., Müller, S., Garcia-Gonzalez, E., Poppinga, L., Süssmuth, R. D., & Genersch, E. (2014). Production of the Catechol Type Siderophore Bacillibactin by the Honey Bee Pathogen Paenibacillus larvae. *PLoS ONE*, *9*(9), e108272. https://doi.org/10.1371/journal.pone.0108272

Heyndrickx, M., Vandemeulebroecke, K., Hoste, B., Janssen, P., Kersters, K., De Vos, P., ... Berkeley, R. C. W. (1996). Reclassification of Paenibacillus (formerly Bacillus) pulvifaciens (Nakamura 1984) Ash et al. 1994, a Later Subjective Synonym of Paenibacillus (formerly Bacillus) larvae (White 1906) Ash et al. 1994, as a Subspecies of P. larvae, with Emended Description. *International Journal of Systematic Bacteriology*, *46*(1), 270–279. https://doi.org/10.1099/00207713-46-1-270

Kagawa, W., Sagawa, T., Niki, H., & Kurumizaka, H. (2011). Structural basis for the DNA-binding activity of the bacterial β-propeller protein YncE. *Acta Crystallographica Section D: Biological Crystallography*, *67*(12), 1045–1053. https://doi.org/10.1107/S0907444911045033

Katoh, K., & Standley, D. M. (2013). MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Molecular Biology and Evolution*, *30*(4), 772–780. https://doi.org/10.1093/molbev/mst010

Kemperman, R., Kuipers, A., Karsens, H., Nauta, A., Kuipers, O., & Kok, J. (2003). Identification and Characterization of Two Novel Clostridial Bacteriocins, Circularin A and Closticin 574. *Applied and Environmental Microbiology*, *69*(3), 1589–1597. https://doi.org/10.1128/AEM.69.3.1589-1597.2003

Khan, A., Burmeister, A. R., & Wahl, L. M. (2020). Evolution along the parasitism-mutualism continuum determines the genetic repertoire of prophages. *PLoS Computational Biology*, *16*(12), 1–19. https://doi.org/10.1371/journal.pcbi.1008482

Kirn, T. J., Jude, B. A., & Taylor, R. K. (2005). A colonization factor links Vibrio cholerae environmental survival and human infection. *Nature*. https://doi.org/10.1038/nature04249

Konno, K., & Mitsuhashi, W. (2019). The peritrophic membrane as a target of proteins that play important roles in plant defense and microbial attack. *Journal of Insect Physiology*, *117*(June), 103912. https://doi.org/10.1016/j.jinsphys.2019.103912

Koskella, B., & Brockhurst, M. A. (2014). Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS Microbiology Reviews*, *38*(5), 916–931. https://doi.org/10.1111/1574-6976.12072

Kyrkou, I., Carstens, A. B., Ellegaard-Jensen, L., Kot, W., Zervas, A., Djurhuus, A. M., ... Hansen, L. H. (2019). Expanding the diversity of myoviridae phages infecting lactobacillus plantarum—A novel lineage of lactobacillus phages comprising five new members. *Viruses*, *11*(7). https://doi.org/10.3390/v11070611

Lewis, J. A. (2001). Control of directionality in integrase-mediated recombination: examination of recombination directionality factors (RDFs) including Xis and Cox proteins. *Nucleic Acids Research*, *29*(11), 2205–2216. https://doi.org/10.1093/nar/29.11.2205

Li, G., Shen, M., Lu, S., Le, S., Tan, Y., Wang, J., ... Li, M. (2016). Identification and characterization of the HicAB toxin-antitoxin system in the opportunistic pathogen Pseudomonas aeruginosa. *Toxins*, *8*(4), 1–12.

https://doi.org/10.3390/toxins8040113

Magaziner, S. J., Zeng, Z., Chen, B., & Salmond, G. P. C. (2019). The Prophages of Citrobacter rodentium Represent a Conserved Family of Horizontally Acquired Mobile Genetic Elements Associated with Enteric Evolution towards. *Journal of Bacteriology*, (January), 1–26. https://doi.org/doi.org/10.1128/JB.00638-18

Mahony, J., Alqarni, M., Stockdale, S., & Spinelli, S. (2016). Functional and structural dissection of the tape measure protein of lactococcal phage TP901-1. *Nature Publishing Group*, (August), 1–10. https://doi.org/10.1038/srep36667

McHugh, J. P., Rodríguez-Quiñones, F., Abdul-Tehrani, H., Svistunenko, D. A., Poole, R. K., Cooper, C. E., & Andrews, S. C. (2003). Global iron-dependent gene regulation in Escherichia coli: A new mechanism for iron homeostasis. *Journal of Biological Chemistry*, *278*(32), 29478–29486. https://doi.org/10.1074/jbc.M303381200

Merrill, B.D, Fajardo, C. ., Hilton, J. ., Payne, A. ., Ward, A. ., Walker, J. ., ... Al., E. (2018). Complete genome sequences of eighteen Paenibacillus larvae phages. *Genome Announc*.

Merrill, Bryan D, Fajardo, C. P., Hilton, J. A., Payne, A. M., Ward, A. T., Walker, J. K., ... Tsourkas, P. K. (2018). Complete Genome Sequences of 18 Paenibacillus larvae Phages from the Western United States. *Microbiology Resource Announcements*, *7*(13), 4–6. https://doi.org/10.1128/MRA.00966-18

Müller, S., Garcia-Gonzalez, E., Genersch, E., & Süssmuth, R. D. (2015). Involvement of secondary metabolites in the pathogenesis of the American foulbrood of honey bees caused by Paenibacillus larvae. *Natural Product Reports*, *32*(6), 765–778. https://doi.org/10.1039/C4NP00158C

Nakajima, C., Okayama, A., Sakogawa, T., Nakamura, A., & Hayama, T. (1997). Disposition of Ampicillin in Honeybees and Hives. *Journal of Veterinary Medical Science*, *59*(9), 765–767. https://doi.org/10.1292/jvms.59.765

Nakamura, K., Okumura, K., Harada, M., Okamoto, M., Okura, M., & Takamatsu, D. (2021). Peritrophic matrix-degrading proteins are dispensable virulence factors in a virulent Melissococcus plutonius strain. *Scientific Reports*, *11*(1), 1–13. https://doi.org/10.1038/s41598-021-88302-8

O'Brien, A. D., Newland, J. W., Miller, S. F., Holmes, R. K., Smith, H. W., & Formal, S. B. (1984). Shiga-Like Toxin-Converting Phages from Escherichia coli Strains That Cause Hemorrhagic Colitis or Infantile Diarrhea. *Science*, *226*(4675), 694–696. https://doi.org/10.1126/science.6387911

Oliveira, A., Melo, L. D. R., Kropinski, A. M., & Azeredo, J. (2013). Complete Genome Sequence of the Broad-Host-Range Paenibacillus larvae Phage phiIBB_Pl23. *Genome Announcements*, *1*(5), e00438-13-e00438-13. https://doi.org/10.1128/genomeA.00438-13

Oliveira, H., Sampaio, M., Melo, L. D. R., Dias, O., Pope, W. H., Hatfull, G. F., & Azeredo, J. (2019). Staphylococci phages display vast genomic diversity and evolutionary relationships. *BMC Genomics*, *20*(1), 357. https://doi.org/10.1186/s12864-019-5647-8

Olszak, T., Latka, A., Roszniowski, B., Valvano, M. A., & Drulis-Kawa, Z. (2017). Phage Life Cycles Behind Bacterial Biodiversity. *Current Medicinal Chemistry*, *24*(36), 3987–4001. https://doi.org/10.2174/0929867324666170413100136

Papić, B. of the G. P. S. of P. larvae and O. I. of A. F. U. a S. wgMLST S., Diricks, M., & Kušar, D. (2021). Analysis of the Global Population Structure of Paenibacillus larvae and Outbreak Investigation of American Foulbrood Using a Stable wgMLST Scheme. *Frontiers in Veterinary Science*, *8*(February), 1–15. https://doi.org/10.3389/fvets.2021.582677

Pell, L. G., Kanelis, V., Donaldson, L. W., Lynne Howell, P., & Davidson, A. R. (2009). The phage major tail protein structure reveals a common evolution for long-tailed phages and the type VI bacterial secretion system. *Proceedings of the National Academy of Sciences*, *106*(11), 4160–4165. https://doi.org/10.1073/pnas.0900044106

Popoff, M. R. (2011). Epsilon toxin: A fascinating pore-forming toxin. *FEBS Journal*, *278*(23), 4602–4615. https://doi.org/10.1111/j.1742-4658.2011.08145.x

Rauch, S., Ashiralieva, A., Hedtke, K., & Genersch, E. (2009). Negative Correlation between Individual-Insect-Level Virulence and Colony-Level Virulence of Paenibacillus larvae, the Etiological Agent of American Foulbrood of Honeybees. *Applied and Environmental Microbiology*, *75*(10), 3344–3347. https://doi.org/10.1128/AEM.02839-08

Regni, C., Tipton, P. A., & Beamer, L. J. (2002). Crystal structure of PMM/PGM: An enzyme in the biosynthetic pathway of P. aeruginosa virulence factors. *Structure*, *10*(2), 269–279. https://doi.org/10.1016/S0969-2126(02)00705-0

Reybroeck, W., Daeseleire, E., De Brabander, H. F., & Herman, L. (2012). Antimicrobials in beekeeping. *Veterinary Microbiology*, *158*(1–2), 1–11. https://doi.org/10.1016/j.vetmic.2012.01.012

Ribeiro, H. G., Melo, L. D. R., Oliveira, H., Boon, M., Lavigne, R., Noben, J.-P., ... Oliveira, A. (2019). Characterization of a new podovirus infecting Paenibacillus larvae. *Scientific Reports*, *9*(1), 20355. https://doi.org/10.1038/s41598-019-56699-y

Rosa, D. P. De, Pérez, J. J., Rosa, D., Cossio-bayugar, R., Miranda-miranda, E., Lozano, L., & Bravo-díaz, M. A. (2015). Complete Genome Sequence of Paenibacillus larvae MEX14 , Isolated from Honey Bee Larvae from the Xochimilco Quarter in Mexico City, *3*(4), 90914. https://doi.org/10.1128/genomeA.00968-15.Copyright

Ruiz-cruz, S., Parlindungan, E., Garzon, A. E., Alqarni, M., Lugli, G. A., Ventura, M., ... Mahony, J. (2020). Lysogenization of a Lactococcal Host with Three Distinct Temperate Phages Provides Homologous and Heterologous Phage Resistance. *Microorganisms*, 1–14. https://doi.org/10.3390/microorganisms8111685

Sheflo, M. A., Gardner, A. V, Merrill, B. D., Fisher, J. N. B., Lunt, B. L., Breakwell, D. P., & Grose, J. H. (2013). Correction for Sheflo et al ., Complete Genome Sequences of Five Brevibacillus laterosporus Bacteriophages, *3*(5), 2015.

Simanshu, D. K., Yamaguchi, Y., Park, J.-H., Inouye, M., & Patel, D. J. (2013). Structural Basis of mRNA Recognition and Cleavage by Toxin MazF and Its Regulation by Antitoxin MazE in Bacillus subtilis. *Molecular Cell*, *52*(3), 447–458. https://doi.org/10.1016/j.molcel.2013.09.006

Slavicek, J. M. (2012). Baculovirus Enhancins and Their Role in Viral Pathogenicity. In *Molecular Virology*. InTech. https://doi.org/10.5772/33285

Solomon, J. M., & Grossman, A. D. (1996). Who's competent and when: regulation of natural genetic competence in bacteria. *Trends in Genetics*, *12*(4), 150–155. https://doi.org/10.1016/0168-9525(96)10014-7

Spaan, A. N., van Strijp, J. A. G., & Torres, V. J. (2017). Leukocidins: staphylococcal bi-component pore-forming toxins find their receptors. *Nature Reviews Microbiology*, *15*(7), 435–447. https://doi.org/10.1038/nrmicro.2017.27

Stamereilers, C., Fajardo, C., Walker, J., Mendez, K., Castro-Nallar, E., Grose, J., ... Tsourkas, P. (2018). Genomic Analysis of 48 Paenibacillus larvae Bacteriophages. *Viruses*, *10*(7), 377. https://doi.org/10.3390/v10070377

Stanczak-Mrozek, K. I., Laing, K. G., & Lindsay, J. A. (2017). Resistance gene transfer: Induction of transducing phage by sub-inhibitory concentrations of antimicrobials is not correlated to induction of lytic phage. *Journal of Antimicrobial Chemotherapy*, *72*(6), 1624–1631. https://doi.org/10.1093/jac/dkx056

Taylor, V. L., Fitzpatrick, A. D., Islam, Z., & Maxwell, K. L. (2019). The Diverse Impacts of Phage Morons on Bacterial Fitness and Virulence. *Advances in Virus Research*, *103*, 1–31. https://doi.org/10.1016/bs.aivir.2018.08.001

Thomet, M., Trautwetter, A., Ermel, G., & Blanco, C. (2019). Characterization of HicAB toxin-antitoxin module of Sinorhizobium meliloti. *BMC Microbiology*, *19*(1), 10. https://doi.org/10.1186/s12866-018-1382-6

Touchon, M., Bernheim, A., & Rocha, E. P. C. (2016). Genetic and life-history traits associated with the distribution of

prophages in bacteria. *The ISME Journal*, *10*(11), 2744–2754. https://doi.org/10.1038/ismej.2016.47

Touchon, M., Bobay, L. M., & Rocha, E. P. C. (2014). The chromosomal accommodation and domestication of mobile genetic elements. *Current Opinion in Microbiology*, *22*, 22–29. https://doi.org/10.1016/j.mib.2014.09.010

Touchon, M., Moura de Sousa, J. A., & Rocha, E. P. (2017). Embracing the enemy: The diversification of microbial gene repertoires by phage-mediated horizontal gene transfer. *Current Opinion in Microbiology*, *38*, 66–73. https://doi.org/10.1016/j.mib.2017.04.010

Toussaint, A., & Rice, P. A. (2017). Transposable phages, DNA reorganization and transfer. *Current Opinion in Microbiology*, *38*, 88–94. https://doi.org/10.1016/j.mib.2017.04.009

Tsourkas, P. K. (2020). Paenibacillus larvae bacteriophages: obscure past, promising future. *Microbial Genomics*, *6*(2). https://doi.org/10.1099/mgen.0.000329

Tsourkas, P. K., Yost, D. G., Krohn, A., LeBlanc, L., Zhang, A., Stamereilers, C., & Amy, P. S. (2015). Complete Genome Sequences of Nine Phages Capable of Infecting Paenibacillus larvae , the Causative Agent of American Foulbrood Disease in Honeybees. *Genome Announcements*, *3*(5), e01120-15. https://doi.org/10.1128/genomeA.01120-15

Unterholzner, S. J., Poppenberger, B., & Rozhon, W. (2013). Toxin–antitoxin systems. *Mobile Genetic Elements*, *3*(5), e26219. https://doi.org/10.4161/mge.26219

Veesler, D., & Cambillau, C. (2011). A Common Evolutionary Origin for Tailed-Bacteriophage Functional Modules and Bacterial Machineries. *Microbiology and Molecular Biology Reviews*, *75*(3), 423–433. https://doi.org/10.1128/mmbr.00014-11

Wachino, J., Jin, W., Kimura, K., & Arakawa, Y. (2019). Intercellular Transfer of Chromosomal Antimicrobial Resistance Genes between Acinetobacter baumannii Strains Mediated by Prophages. *Antimicrobial Agents and Chemotherapy*, *63*(8), 1–16. https://doi.org/doi.org/10.1128/AAC .00334-19

Waldor, M. K., & Mekalanos, J. J. (1996). Lysogenic Conversion by a Filamentous Phage Encoding Cholera Toxin. *Science*, *272*(5270), 1910–1914. https://doi.org/10.1126/science.272.5270.1910

Walker, J. K., Merrill, B. D., Berg, J. A., Dhalai, A., Dingman, D. W., Fajardo, C. P., ... Tsourkas, P. K. (2018). Complete genome sequences of Paenibacillus larvae phages BN12, Dragolir, Kiel007, Leyra, Likha, Pagassa, PBL1c, and Tadhana. *Genome Announcements*, *6*(24), 12–13. https://doi.org/10.1128/genomeA.01602-17

Wang, M., Zeng, Z., Jiang, F., Zheng, Y., Shen, H., Macedo, N., ... Li, G. (2020). Role of enterotoxigenic <scp> *Escherichia coli* </scp> prophage in spreading antibiotic resistance in a porcine-derived environment. *Environmental Microbiology*, *22*(12), 4974–4984. https://doi.org/10.1111/1462-2920.15084

Wen, Y., Behiels, E., & Devreese, B. (2014). Toxin-Antitoxin systems: Their role in persistence, biofilm formation, and pathogenicity. *Pathogens and Disease*, *70*(3), 240–249. https://doi.org/10.1111/2049-632X.12145

Xia, G., & Wolz, C. (2014). Phages of Staphylococcus aureus and their impact on host evolution. *Infection, Genetics and Evolution*, *21*, 593–601. https://doi.org/10.1016/j.meegid.2013.04.022

Xin, W., & Wang, J. (2019). Clostridium perfringens epsilon toxin: Toxic effects and mechanisms of action. *Biosafety and Health*, *1*(2), 71–75. https://doi.org/10.1016/j.bsheal.2019.09.004

Yost, D. G., Chang, C., LeBlanc, L., Cassin, E., Peterman, C., Rai, P., ... Tsourkas, P. K. (2018). Complete Genome Sequences of Paenibacillus larvae Phages Halcyone, Heath, Scottie, and Unity from Las Vegas, Nevada. *Microbiology Resource Announcements*, *7*(12), 1–2. https://doi.org/10.1128/MRA.00977-18

Yost, D. G., Tsourkas, P., & Amy, P. S. (2016). Experimental bacteriophage treatment of honeybees (Apis mellifera) infected

with Paenibacillus larvae, the causative agent of American foulbrood disease. *Bacteriophage*, *6*(1), e1122698. https://doi.org/10.1080/21597081.2015.1122698

Yue, D., Nordhoff, M., Wieler, L. H., & Genersch, E. (2008). Fluorescence in situ hybridization (FISH) analysis of the interactions between honeybee larvae and Paenibacillus larvae, the causative agent of American foulbrood of honeybees (Apis mellifera). *Environmental Microbiology*, *10*(6), 1612–1620. https://doi.org/10.1111/j.1462-2920.2008.01579.x

Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J., & Wishart, D. S. (2011). PHAST : A Fast Phage Search Tool, *39*(June), 347–352. https://doi.org/10.1093/nar/gkr485

CHAPTER 7: CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 Final conclusions

Phages have been considered promising approached to mitigate the increase of antimicrobial resistance (AMR) bacteria. Although the use of antibiotics to treat AFB in Europe is forbidden, they still can be used in many countries of America and Asia, under veterinary prescription. In addition to having no effect on *P. larvae* spores, antibiotics contribute to decrease the diversity of bee commensal flora, essential to maintain hive health, and remains as residues in hive products that, if consumed, can harm human health. In the EU, infection control is compulsorily done by burning contaminated hives, and thus, outbreaks, contribute to important economic and environment losses. Alternative and sustainable ways to control *P. larvae*, such as the use of phages, has been explored.

This work firstly aimed to develop a phage-based product to control and prevent AFB but also intended to demonstrate that phages could be delivered to larvae through feeding by adult bees mainly if they are protected from hive conditions.

The biodistribution and bioavailability assay with T7 phage, confirmed the success of providing phages to adult bees and getting them into larvae. Phage presence in larvae tissues were observed by culture assays, qPCR and immunohistochemical analysis. This oral administration represents an advantage to phage delivery, reducing waste and phage losses and being readily to use (easily included in hive management). However, the assay also indicated that phages might need to be protected from harsh hive environment (mainly from RJ) to increase therapeutic efficacy in larvae midgut.

To explore the *in vivo* performance, new *P. larvae*- specific phages were isolated and characterized – API480, API46 and API59 – but only the first one was further evaluated *in vivo* and encapsulated in a pH-responsive polymer. The biodistribution and bioavailability of free or encapsulated phage had similar results in larvae. Despite reaching larvae, the concentration was not higher when the phage was protected, and this was mainly due to the type of encapsulation used, highly difficult to disperse in watery solutions with pH < 7. Nevertheless, the polymer was able to protect API480 viability in crops, where the pH is low.

The evaluation of phage performance in infected larvae, revealing a bacterial reduction of about 2.0 Log CFU/larva in 47% of the treated insects, when a time-lapsed administration using a second phage (CEB16, active against API480-resistant mutants) was applied. Even though providing encouraging results, they suggest the use of virulent phages to avoid superinfection exclusion event. Genetic manipulation can thus be an important strategy to use.

Studies on the impact of *P. larvae* prophages in *P. larvae* fitness or toxicity were relevant and innovative from the ecological point of view. Most of the analysed bacteria contained intact prophages in

their genome, that seemed to influence hosts by providing, for example, TA-systems, and toxins (enhancin-like protein, N-acetylglucosamine (GlcNAc)-binding protein A (GbpA), LukF-PV or EtxB) that increase the bacteria virulence, proteins related to nutrient acquisition such as SufB and NifU that help in the iron capture or metabolism like phosphomannomutases, proteins associated to HGT and external DNA internalization and transport (transposases and protein ComEC/Rec2). Moreover, the idea that specific virulence or fitness genes from prophages are some way connected with the host ERIC genotypes was supported, especially for ERIC V genotype. The number of new prophages analysed here contributed to update the information on phage genomic and taxonomic diversity.

Overall results achieved in the scope of this work were a simple and initial step towards finding *P. larvae* phage solutions for beekeeping. Yet, further studies are needed to make such a product a reality, saving millions of bees and consequently decreasing the impact on the environment and ecosystem.

7.2 Future perspectives

Despite of the important steps and main conclusions obtained, more has to be done in order to implement the best phage solutions for beekeeping.

Based on the small number of phages known, it is necessary to keep isolating phages, characterizing, and testing them *in silico* (e.g.: genomic, proteomic), *in vitro* (e.g.: lytic spectra, life cycle), and *in vivo* (e.g.: reared lab-larvae or hives), to increase the phage diversity and availability for therapeutic options. Moreover, the sequencing of new *P. larvae* strains will allow a more realistic description of their prophages and respective genes, to define those predominantly present in the *P. larvae* populations and involved in outbreaks. The analysis of host genomes will also clarify the main types of *P. larvae* anti-phage defence systems supporting the design of strategies to overcome them.

The isolation of exclusively virulent *P. larvae* phages shall be pursued, to avoid concerns related to superinfection immunity leading to bacterial resistance or the transfer of toxic genes from phage to host.

However, if only temperate *P. larvae* phages are available, their genetic manipulation to remove undesirable genes shall be considered. In addition, its performance can be improved by adding genes that enhance its action or recognition.

The study the *P. larvae* phage host receptors clarifying how mutations in hosts modify the receptors and allowing the mixture of phages targeting distinct cell molecules will be definitely important. Phage receptor–binding proteins also should be investigated to clarify and find whether *P. larvae* phages have the ability to bind specifically also to spores and not only to vegetative cells. The phage protection to hive conditions shall be improved and the wise selection of phages for cocktails shall be done.

Additionally, the study of how *P. larvae* phages change the proteome of larvae, genomic expression or microbiome after reaching the midgut will bring important information to study larvae response to therapy.

For phage production will be important to have cured hosts, without any prophage, to avoid crossed phages contamination.

This work provided important directions to be further explored and investigated aiming AFB control.