

Host Immunology and Pathogen-Bacteriophage
interactions in Black Soldier Fly Larvae (BSFL;
Hermetia illucens)

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Abstract

The Black Soldier Fly (*Hermetia illucens*) has emerged as a sustainable bioconversion species with considerable potential for a circular economy. While extensively studied for waste management and protein production, its innate immune system remains comparatively underexplored. This thesis aims to establish and optimise experimental and molecular approaches to investigate host immune responses and bacteriophage-pathogen interactions in *H. illucens*, providing new insight into its immunological potential and implications for sustainable disease control.

Methodological optimisation included refining larval rearing conditions, bioactive administration, and RNA extraction techniques tailored to the physiological characteristics of *H. illucens*. Using pathogen-associated molecular patterns (PAMPS), lipopolysaccharide (LPS) and polyinosinic:polycytidylic [Poly (I:C)], the dose-dependent induction of pattern-recognition receptors and antimicrobial peptide genes was quantified through qPCR analysis. Responses showed thresholds and paralogue-specific expression profiles, indicating scalable innate activation.

A directional poly (A) RNA-seq transcriptome was generated from pooled whole-body larvae and subjected to genome-guided, orthology-informed annotation to produce a curated immune gene database. Canonical innate immune pathways, including Toll, IMD, JAK/STAT and JNK signalling, were characterised through Gene Ontology, KEGG, Reactome, and STRING protein-protein interaction analyses, providing a comprehensive molecular framework for future functional and comparative studies.

Finally, prophylactic administration of bacteriophage SPFM14 significantly reduced *Salmonella enterica* serovar Typhimurium (SL1344) colonisation in *H. illucens*, supporting the potential of phage-based biocontrol as an alternative to antibiotics in insect production systems. Time-course experiments further demonstrated rapid phage shedding from the larval gut, providing important context for interpreting in vivo phage persistence and bacteriophage-mediated bacterial control.

Ethics

This thesis was carried out in accordance with the UK Scientific Procedures Act. All chapters gained prior approval by the University of Stirling Animal Welfare and Ethical Review Body (AWERB 2023 12442 11402).

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Chapter 1. Introduction to the Biology, Industrial Applications, and Regulatory Frameworks of the Black Soldier Fly (*Hermetia illucens*) in Sustainable Feed and Waste Systems

1.1. Introduction to the industrial applications of The Black Soldier Fly (*Hermetia illucens*)

Black Soldier Fly Larvae (BSFL) (*Hermetia illucens*) have become a rapidly growing area of research, due to their multi-chain commercial significance. BSF are voracious consumers of almost all decaying material and can convert large quantities of food waste into high-quality proteins and lipids (Schiavone, De Marco, & Gai, 2023) (Wang & Shelomi, 2017). This makes them a promising sustainable alternative to traditional animal feed ingredients such as fishmeal, which contributes to overfishing, and soymeal, whose production is a major driver of deforestation (Henry, Gasco, Piccolo, & Fountoulaki, 2023) (Van Huis, 2021). Studies have already highlighted the benefits of BSFL inclusion in the diets of various animals within aquaculture and agriculture industries, with numerous reports of improved immune health, growth performance, and welfare status (Gasco, Finke, & Van Huis, 2020) (Fawole J. , 2022) (Tippayadara, et al., 2021).

The global Black Soldier Fly industry has gained momentum in response to growing concerns around food insecurity, waste accumulation, and the environmental footprint of traditional animal production systems (FAO, 2021) (Parodi, de Boer, Slegers, & van Zanten, 2024). BSFL can be reared on a wide range of organic waste streams, including agricultural residues, food scraps, and manure, presenting a unique opportunity to close nutrient loops and recover value from materials that would otherwise be lost to landfills (Xiao, Jin, Li, Zhang, & Liu, 2022) (Lalander, Diener, Zurbrügg, & Vinnerås, 2019). As the demand for sustainable feed and fertiliser sources continues to rise, BSFL are increasingly being adopted in circular bioeconomy models, with applications spanning livestock and aquafeed production, organic waste management, bioplastics, biodiesel, pharmaceuticals, and soil enrichment (Gligorescu, Toepfl, Heinz, & Czerny, 2022). Recent advances in insect farming technology, genetics, and microbial engineering have further expanded the scope of BSFL use, although challenges around scalability, regulation, and cost remain (Generalovic T. N., et al., 2021).

In addition to waste management and protein production, BSFL also offer other industrial applications, such as fertiliser from their nutrient-rich frass (Beesigamukama, et al., 2020), and bioactive compounds like antimicrobial peptides (AMPs), which have promising potential for pharmaceutical applications due to their immunomodulatory properties (Park, Kim, & Yoe, 2015) (Van Moll, et al., 2022)

This chapter will explore the biological, commercial, environmental, and regulatory, considerations surrounding the use of BSFL in modern industry, highlighting both their potential and limitations as a sustainable bioconversion tool.

1.2. The Black Soldier Fly (*Hermetia illucens*)

The Black Soldier Fly (BSF), *Hermetia illucens* (Diptera: Stratiomyidae) (Table 1.1; Figure 1.1), is a species that is native to the Neotropical Realm, but has since populated across all continents, particularly across temperate climates (Diener, et al., 2011). They can range between 15-20mm in length as adults, and have characteristic black bodies with metallic reflections, ranging from blue and green on their thorax. They have a translucent abdominal segment (see Figure 1.1) and at times, have a reddish tint to the end of their abdomen. They don't sting or bite, and there are no reports of Black Soldier Flies acting as a vector for disease (Wang & Shelomi, 2017). Acoustic signalling, generated by the wings of the male fly, is considered vital for female acceptance (Giunti, Campolo, Laudani, & Palmeri, 2018). Females can lay between 500-900 eggs, laying them in crevices near to a food source, such as compost or manure (Perma, Julita, F, & Putra, 2020), (Booth & Sheppard, 1984) (Chia S. , et al., 2018). Eggs hatch around 4 days after oviposition, with the complete life cycle of *H. illucens* lasting approximately 45 days (Booth & Sheppard, 1984). This consists of five main stages: egg, larval, prepupal, pupal, and adult stages (Da Silva & Hesselberg, 2019). Emerging larvae begin feeding immediately and measure from 1mm, to 20mm once fully matured. The larval phase consists of six instars, which are largely spent consuming a wide variety of organic matter, such as animal manure, food waste, and discarded grains, among others. Once larvae reach the 3rd instar, consumption rates are significantly increased (Liu, Wang, &

Yao, 2019), and at the 6th instar, they cease to eat and empty their digestive tract, initiating the prepupal stage. Prepupae migrate from their food source to an area that is dry, and dark to metamorphose into pupae, taking around 7-10 days. Morphological changes at this stage, include the labrum bending, allowing them to ‘hook and pull’, to a suitable pupation site. Once entering the pupal stage, they will lay dormant for at least 8 days until the adult emerges. The emergence of the adult generally takes two weeks but can be up to 5 months if their optimal conditions are not met. It was expected that adult BSF only need access to water and will not feed, since they rely on fat stores gained from their larval stage (Sheppard, Newton, Thompson, & Savage, 1994) (Sheppard, Tomberlin, Joyce, Kiser, & Sumner, 2002) (Tomberlin & Sheppard, 2002) (Tomberlin, Sheppard, & Joyce, 2002). However, reports of increased longevity have been reported following sugar intake, as well as increased oviposition and longevity with a protein-rich diet (Nakamura, Ichiki, Shimoda, & Morioka, 2016) (Bertinetti, Samayoa, & Hwang, 2019). In addition to their fascinating biological traits and adaptability, BSFL have attracted increasing attention due to their broad potential across multiple industrial sectors, including waste management, animal nutrition, and biotechnology.

Table 1.1 Taxonomic classification of the Black Soldier Fly (*Hermetia illucens*) from phylum species level, highlighting its position within the order Diptera and family Stratiomyidae

Phylum	Arthropoda
Class	Insecta
Order	Diptera
Suborder	Brachycera
Superfamily	Stratiomyoidea
Family	Stratiomyidae
Subfamily	Hermetiinae
Genus	<i>Hermetia</i>
Species	<i>H. illucens</i>

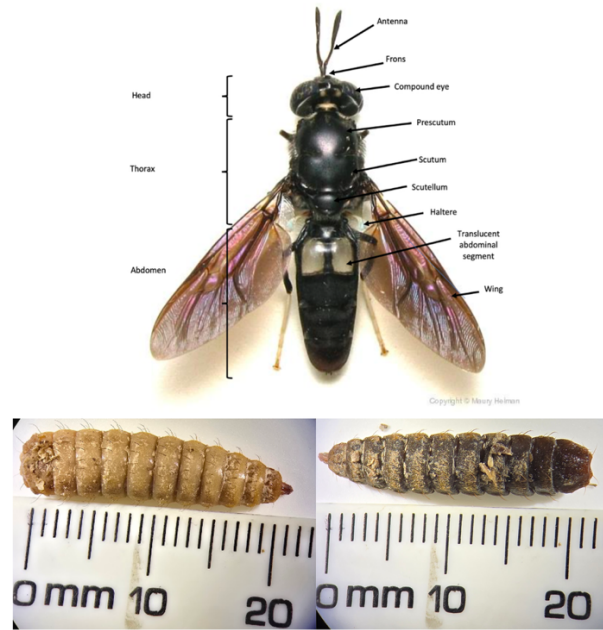


Figure 1.1 Adult and larval morphology of the black soldier fly *Hermetia illucens*. Labelled diagram of an adult Black Soldier Fly (BSF). Image © Maury Heiman. Modified and annotated by the author. The bottom images show a late-instar larva (left) and a prepupa (right), demonstrating characteristic size, segmentation, and darkening of the cuticle during the transition to the pupal stage

1.3. Black Soldier Fly Larvae for Sustainable Organic Waste Management

With a rapidly growing population and continued urbanisation, Black Soldier Fly Larvae (BSFL) have become an emerging vermicomposting model for alternative treatment for food waste and agricultural waste, i.e., manure (Bogner, et al., 2008) (Elissen, 2007). The biochemical properties of the Black Soldier Fly make it an efficient decomposer of organic materials. High levels of digestive enzymes such as amylase, lipase, and proteases within the gut and salivary glands of BSFL make them extremely efficient at digesting various kinds of food (Sheppard, Tomberlin, Joyce, Kiser, & Sumner, 2002). Every year, more than 2.1 million tons of municipal waste are generated, putting extreme pressure on governments, particularly those from low- and middle-income countries, to provide adequate waste management services. As the population reaches the expected 9.8 billion by 2050, the countries expected to contribute the most to the total growth are: India, Nigeria, the Democratic Republic of the Congo, Pakistan, Ethiopia, the United Republic of Tanzania, the United States of America, Uganda and

Indonesia (Kim, et al., 2011). Due to low collection rates, the total amount of bio-waste (organic material) produced in low- and middle-income settings reaches between 50-70%, compared to 20-40% obtained in high-income settings. With no valid solution in developing countries, the global survey by the United Nations Development Program (UNDP) highlighted this as the second most serious issue after unemployment, impacting human health, the environment, and hindering economic progression (United Nations, 2017) (United Nations, 1997). Furthermore, inadequate disposal of food waste contributes to the release of methane into the environment, with landfill and wastewater accounting for approximately 18% of the global anthropogenic methane emissions (Iswa & Wilson, 2015). Even with current practices in recycling and biodegradables, landfilling and incineration are responsible for toxins, leachate, and greenhouse gases. Thus, implementing new, cost-effective waste treatment technology is gaining momentum within research. BSF farming rapidly converts low-value organic material, into macromolecules with high nutritional value, presenting key opportunities to valorise waste and provide feed for livestock, as well as being the starting material for several other commercial products (Lim, Lee, & Wu, 2016), (Setti, et al., 2019) (Leong, Rahman, Kutty, Malakahmad, & Tan, 2016), (Li, et al., 2011). By taking advantage of the closed-loop cycle of natural ecosystems, Black Soldier Fly Larvae can recycle the vital nutrients that would otherwise be locked up by consuming biowaste, converting it into larval biomass, and leaving a substrate residue with characteristics like immature compost. Thus, this emerging technology can offer an ecologically sustainable waste management process, requiring fewer valuable resources such as land and water per unit of protein than protein crops (Wang & Shelomi, 2017). Each larva can consume up to 200mg of food waste per day and is also capable of removing certain toxic substances from waste streams (Nyakeri, Ayieko, Amimo, Salum, & Ogola, 2019). Various life-history traits of BSFL are controlled by the nutritional quality of the substrates; therefore, research has been devoted to optimising the rearing performance of BSFL and determining if certain contaminants and/or different waste streams affect the life cycle and bioremediation capabilities of larvae.

1.4. Maintaining Black Soldier Fly Larvae

The rearing process must meet the conditions that are suitable for proper development of BSF. The time spent in each stage of its life cycle largely depends on physical conditions, food availability and quality of the substrate (Diener, Zurbrügg, & Tockner, 2009) (Tomberlin, Sheppard, & Joyce, 2002) (Nguyen, Tomberlin, & VanLaerhoven, 2013). Optimal growth and development (e.g., oviposition) depend on several abiotic factors such as temperature, humidity, moisture, pH, and light.

1.5. Optimal temperature for rearing BSFL

Black Soldier Flies (BSF) are a eurythermal species that can tolerate wide ranges of temperature (15-47°C); however, it is important to optimise these temperatures for efficient growth, bioconversion, and reproduction. For optimal egg incubation, neonate care, and larval development, maintain a dark environment 24 hours daily with a consistent temperature of $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with a relative humidity of 70%. Adult black soldier flies are most active and achieve optimal mating and oviposition between 26-30°C, with reduced reproductive performance below 24°C and above 35°C (Sheppard, Tomberlin, Joyce, Kiser, & Sumner, 2002).

1.6. Optimal humidity for rearing BSFL

Various authors have reported 60-70% humidity yields the best results in *Hermetia illucens* larvae. An increase in relative humidity is directly correlated with lower survival rates. Relative humidity at 25, 40 and 70% had mortality rates of 62, 26, and 3%, respectively (Holmes, Vanlaerhoven, & Tomberlin, 2012). In adult flies, humidity supported mating and oviposition between 30-90%, however, some reports of higher humidity increased the risk of fungal development (Tomberlin, Adler, & Myers, 2009).

1.7. Optimal moisture for rearing BSFL

The presence of excess moisture can affect the growth and survival of BSF (Cheng, Chiu, & Lo, 2017). Larvae have been grown at substrate moisture contents of 90-97%, however, this is often met with decreased survival rates, difficulty in processing waste streams, and reduced body weight than when substrate moisture content is at ~75-80% (Diener, et al., 2011) (Lalander, Ermolaev, Wiklicky, & Vinnerås, 2020). Depending on the waste stream, moisture content may need to be adjusted. Substrate moisture content affects larvae mobility, texture, feed consumption, growth, and survival, and is also linked to variations in microbial activity (Palma, et al., 2018) (Makkar, Tran, Heuzé, & Ankers, 2014). Increased microbial substrate degradation to CO₂, short growth phases, and efficient bioconversion were positively influenced by low substrate moisture contents (Bekker, et al., 2021).

1.8. Optimal pH for rearing BSFL

The lifecycle and survival of BSF is influenced by the pH, with various authors reporting that a pH of 6.0-8.0 is optimum (Meneguz, Gasco, & Tomberlin, 2018). Larval density plays a role in regulating the pH of organic medium, with one study showing that BSF larvae can regulate the pH up to 9.0 for liquid organic leachates (Green & Popa, 2012). Ma et al. found that larval weight was significantly greater when reared on substrates with an initial pH of 6.0-10.0, with lower pH levels (pH 2.0) causing a reduction in larval weight (Ma, et al., 2018).

1.9. Optimal light requirements for rearing BSFL

Black soldier fly larvae are strongly photonegative and perform best in darkness or very low ambient light, and no specific photoperiod is required for development (Giannetti, et al., 2022). In contrast, the adult stage is photophilic, with previous studies showing that BSF mating relies on visual mediation, therefore, irregular light, or low light conditions, decreases BSF reproduction and leads to inconsistent production of fertile eggs (Tingle, Mitchell, & Copeland, 1975). Therefore, to achieve optimal lighting, a combination of natural sunlight and artificial lighting may be required for successful mating such as Quartz-iodine (i.e., halogen lamps), light-emitting diode (LED) lights, and fluorescent lamps (Heussler, et al., 2018) (Nakamura, Ichiki, Shimoda, & Morioka, 2016), (Oonincx, Volk, Diehl, Van Loon, & Belušič, 2016), have all been trialled to determine the effects on oviposition, adult longevity, and the resulting number of young larvae. Approximately 85% of BSF mating occurs in the presence of natural sunlight with an intensity of $110\mu\text{mol m}^2\text{s}^{-1}$ (Park H. , 2016). Insects cannot see light past 700nm; therefore, a range between 450-700nm has been considered optimum for reproductive events in adults (Briscoe & Chittka, 2001) (Zhang, et al., 2010). UV-enriched illumination (LED) lights were found to produce more larvae, as the adult compound eye retina has UV-, blue-, and green-sensitive photoreceptor cells (Oonincx, Volk, Diehl, Van Loon, & Belušič, 2016).

1.10. Contaminant Tolerance and Environmental Remediation

BSFL can be reared on a variety of waste streams and can modify the microflora present within them. Larvae have been found to reduce the quantity of harmful bacteria such as *Escherichia coli* (*E. coli*) and *Salmonella enterica* by secreting potent bactericidal compounds and reducing the production of offensive odours (Erickson, Islam, Sheppard, Liao, & Doyle, 2004) (Lalander, Fidjeland, Diener, Eriksson, & Vinneras, 2015). They are also capable of controlling the population of other flies (e.g., houseflies and blow flies) by repelling their oviposition. This is an advantageous prospect, since these flies are seen as a serious disease vector, particularly in developing countries where inappropriate sanitation accounts for dangerous sources of pathogens (Furman, Young,

& Catts, 1959). Various studies have looked at BSFL reared in substrates with heavy metal accumulation (zinc, lead, and cadmium), none of which led to any significant effect on the larvae; however, the effects of these metals on the gut microbiota of BSFL are relatively uncharacterised (Diener, Zurbrügg, & Tockner, 2015). Another study looked at the impact of larval growth with contaminants, polyethene (PE), polystyrene (PS) and NaCl, which found no changes in larval growth by plastics, but by substrate salinity (Cho, Kim, & Kim, 2020). Attiogbe et al. (Attiogbe, 2019) looked at BSFL reared on a mixture of organic food wastes, chicken faecal matter, and sawdust, spiked with increased concentrations of mercury salt (1000mg, 2000mg, and 3000mg). Waste reduction depended on the number of larvae and the concentration of mercury, but by the 13th day, mercury levels within the compost were below the European Union's (EU's) threshold values of 0.7- 10 mg Hg/kg. However, it should be highlighted that mercury was bio-accumulated by the larvae, which had effects on their growth and physical appearance. Pharmaceuticals such as trimethoprim, roxithromycin, carbamazepine, and pesticides (azoxystrobin and propiconazole) can also be inhibited from spreading into the environment by the larvae. The half-life of all substances was shorter in the BSFL compost, and no bioaccumulation was detected (Lalander, et al., 2016). In addition, another nine antibiotics were efficiently removed over a 6-day period using BSFL vermicomposting, including tetracyclines, sulphonamides, and fluoroquinolones. Abiotic factors such as temperature, pH and total phosphorus negatively influenced the level of residual antibiotics, while positive effects for antibiotic removal were influenced by substrate, total Kjeldahl nitrogen, microbial respiration, and moisture (Zhang, Shen, Wang, & al, 2014). Antibiotic degradation was largely carried out by the intestinal microbiota of BSFL (*Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Basidiomycota*, *Lysurus*, *Trichophyton*, and *Entyloma*), which degraded nearly 97% of tetracycline (TC). This was further demonstrated both *in vivo* and *in vitro* by four larval intestinal isolates, which found increased *tet* gene copy numbers within the gut to allow for efficient degradation of TC (Cai, et al., 2018). Therefore, vermicomposting may be a safe approach to use for antibiotic attenuation, with the potential to inhibit the development and spread of antibiotic resistance from agricultural waste, pesticides, pharmaceutical manufacturing waste, and beyond. However, there are still open-ended questions left to

be answered, to determine any negative impacts these contaminants may have on the life cycle, and performance, of BSFL.

These findings suggest that BSFL are not only robust waste decomposers, but may also serve as biological filters, helping to reduce the environmental impact of contaminated organic streams. While BSFL show promise mitigating contaminants, their broader application in bioconversion depends on how efficiently they process diverse organic substrates, including agricultural and kitchen waste. This is explored in more detail in the following section.

1.11. Organic Waste Processing Through Black Soldier Fly Larvae: Substrate Suitability and System Efficiency

As agricultural production continues to rise alongside a growing global population, waste management is becoming an increasingly difficult task, especially for those in low-income settings. Unmanaged organic waste can give rise to pests, spread diseases and antibiotic resistance, and contribute to global warming through the release of greenhouse gases (GHG). Black Soldier Fly Larvae are now considered a viable bioconversion tool, converting organic waste into protein-rich biomass, which is largely supported by the physiology and morphology of their mid-gut. Enzyme activities are pH dependent; those most abundant in BSFL are amylases, lipases, trypsin- and chymotrypsin- like proteases, which give them the ability to digest different kinds of organic matter (Kim W, 2011). Various substrates have been used to determine BSFL efficiency, including organic kitchen waste, discarded animal feed, animal, and human faecal matter, among others. Fitriana et al. (Fitriana, Laconi, Astuti, & Jayanegara, 2022) performed a meta-analysis to summarise 122 research findings for larvae reared on four different categories: animal feed, food waste, manure, and other substrates. They found that when reared on whole organic substrate, BSFL found it more difficult to process than mass or liquid organic substrate (Surendra, et al., 2020). Animal feed was found to have the most suitable nutrient composition. It showed the highest dry larval weight (DLW) and waste reduction index (WRI), suggesting the substrate was degraded efficiently, consumed, and absorbed into the larvae's biomass. Initial fermentation or

pre-treatment of the substrate further increased the lipid and protein content of BSFL (Wong, et al., 2020) (Wong, et al., 2020). Low survival rates were seen in substrates containing matter with high fibre and undigested nutrient composition. Partial digestion can occur for non-detergent fibre (NDF) and an acid detergent fibre (ADF), as the larvae contain microbes that can produce lignocellulose enzymes; however, utilisation of NDF and ADF was limited (Bonelli, et al., 2020) (Galassi, et al., 2021). The presence of polyunsaturated fatty acids (C18:2n6 and C18:3n3) at high concentrations is recommended within the substrate since they have a positive effect on high polyunsaturated fatty acids in BSFL. Proper drainage, a well-balanced nutrient source, and sufficient abiotic conditions all have positive effects on the efficiency of BSFL composting. If ideal conditions are met, larvae can mature within 2 weeks and could facilitate the bioconversion of 30 tonnes of waste per day while producing 33.3% residue worthy of fertiliser and 7.7% of prepupal biomass for animal feed. However, food shortages, as well as lower temperatures, have extended the larval stage by up to four months, decreasing efficiency and harvesting potential; therefore, optimisation is key for higher yields (Furman, Young, & Catts, 1959). A feeding rate of 1.00g larvae per day produced the highest larval biomass within the shortest development time, with substrate consisting of vegetables, rice, egg and meat (Yuan & Hasan, 2022). Furthermore, BSFL were able to reduce dry matter by 68%, even in the presence of zinc contaminated feed and hostile environmental conditions, allowing the authors to harvest 252 prepupae a day (Diener, Studt Solano, Roa Gutiérrez, Zurbrügg, & Tockner, 2011). This model is already being piloted by several companies, such as Better Origin, which integrate BSFL systems into agricultural settings to upcycle food waste into insect protein.

1.12. Manure Management via Black Soldier Fly Larvae: A Sustainable Waste Reduction Strategy

In addition to food waste, manure represents one of the largest organic waste streams globally. BSFL have demonstrated significant potential for managing these substrates efficiently. The excreta from livestock doesn't readily decompose quickly, and effectively, and can pollute the environment if not handled properly. Increased nitrate levels, bacterial contamination, eutrophication, among others, are all potential outcomes if left untreated. Various studies have looked to determine the efficiency of BSFL in degrading different manure streams, such as poultry, dairy, and swine. BSFL fed a diet of poultry/chicken manure seen higher weight gains and increased waste degradation than those fed on swine and dairy manure. Those reared on pig manure seen a reduction in biomass and nutrient content of the manure with approximately 85% of nitrogen and 75% of phosphorus removed. Cow manure fed BSFL reduced the levels of nitrogen and phosphorus at 30-50% and 61-70%, respectively (Myers, Tomberlin, Lambert, & Kattes, 2008). Mixing faecal sludge with other organic waste made BSFL more efficient waste reducers, most likely due to faecal matter having a very low nutritional value (Diener, et al., 2011). Furthermore, along with the reduction of faecal sludge, BSFL also reduced the pathogen content within the substrate (Lalander, Fidjeland, Diener, Eriksson, & Vinneras, 2015). This is attributed to the vast amount of anti-microbial peptides that the BSF genome can synthesise, allowing them to adapt to environments with dense pathogen and bacterial populations. Larvae fed a single lump amount of human faeces led to slower development but were larger than those on incremental feeds. It was suggested that the larvae increased their feed intake to compensate for nutrient deficiency (Banks, Gibson, & Cameron, 2014). However, Lalander et., al, seen no reduction in prepupal weight in BSFL fed a human faeces diet, which also showed a better amino acid composition compared to poultry or dog feed (Lalander, Diener, Zurbrugg, & Vinnerås, 2019). These studies have highlighted BSFL as a viable solution for manure reduction, co-composting with additional waste streams will ensure BSFL have access to multiple nutritional profiles to perform efficiently. BSFL development was seen to improve significantly when organic waste was added to the faecal sludge mix at a 50:50 ratio.

1.13. Frass as a Fertiliser Product

As BSFL break down organic material and manure, they also leave behind nutrient-rich residues. These by-products, collectively known as frass, have attracted attention for their potential use as sustainable fertilisers. Black Soldier Fly Frass Fertilizer (BSFFF) was evaluated on its performance on maize production, which found those treated with BSFFF had the tallest plants, highest chlorophyll concentrations and macronutrient uptake, performing better than commercial organic and inorganic fertilizers (Beesigamukama, et al., 2020). Vegetable cultivation was seen to have improved performances when treated with composted BSF frass, with sole application boosting the growth, yield, and nutritional quality, which was similar than, or better than commercial organic and mineral fertilizers (Anyega, et al., 2021). These studies highlight the agronomic potential of BSFL frass as a circular, value-added by-product in regenerative agricultural systems.

1.14. BSFL as alternative sources of protein and lipids

While BSFL frass has value as a soil input, the larvae themselves are increasingly valued for their high-protein biomass, which is being positioned as an alternative to conventional animal feed ingredients. The world will have to feed two billion more people in 2050, and by FAO estimates, food production will have to increase by 70% to meet these demands (FAO, 2009). Agriculture has already cleared an area roughly the size of South America as croplands, and an area the size of Africa to raise livestock (Foley, Steinmetz, & Richardson, 2022), causing major disruption to ecosystems all over the globe. Not all crops are grown for human consumption, in fact, 33% of croplands are used for livestock feed production alone. If this could be redirected for human consumption, then there would be enough cropland to feed the 9 billion people, come 2050 (FAO, 2022). Livestock are fed approximately 6kg of plant protein for every kilogram of high-quality protein produced, and demand for meat products are expected to increase by more than 75% in 2050 from current levels (Pimentel & Pimentel, 2003). Therefore, an increased demand for sustainably sourced, high-quality proteins and lipids for animal feed in Aquaculture and livestock industries is needed. Current feed

ingredients, such as soybeans and fishmeal, are becoming increasingly more expensive and have poor carbon footprints; therefore, attention has turned to non-conventional feed ingredients, such as insects, due to their promising potential in increasing farming efficiency and sustainability (de Souza-Vilela, Andrew, & Ruhnke, 2019). Black Soldier Fly Larvae (BSFL), *Hermetia illucens*, are now considered a valuable dietary protein source and may harbour functional properties that encourage animal productivity and health. They are also seen to have major environmental advantages, requiring 50-90% less land than conventional agriculture per kilogram of protein (Farming Connect, 2022). For example, one m² of insect farming can produce up to 750kg of feed, compared to only 0.5kg of soy production on the same amount of land (Figure 1.2) (Better Origin, 2020). Therefore, using BSFL as feed for pets, livestock, and fish is becoming more desirable. By 2030, the global black soldier fly market is expected to reach \$3.4 billion, with a compound annual growth rate (CAGR) of 34.7% during the forecast period of 2020-2030 (Research and Markets, 2022). The 2022 Alltech Global Feed Survey reported that in 2021, international feed tonnage increased to 1.235 billion tonnes, a 2.3% increase from the previous year (Alltech, 2022). Therefore, for insects to be a viable source of replacement feed, significant mass production would be required to meet demands.

The biomass of BSFL is composed of 32-58% proteins and 15-39% lipids (dry weight) and is higher in saturated fats than most insects (Wang & Shelomi, 2017). In addition, they do not accumulate pesticides or mycotoxins and have a similar amino acid profile to that of feed constituents. In this context, they are a valuable source to produce animal feed for livestock, with initial investigations showing promising results (Barragan-Fonseca, Dicke, & van Loon, 2017), (Makkar, Tran, Heuzé, & Ankers, 2014), (Wang & Shelomi, 2017), (Sánchez-Muros, Barroso, & Manzano-Agugliaro, 2014). Other BSFL constituents such as Lauric acid, antimicrobial peptides, and chitin are also considered valuable feed ingredients for animal health (Gasco, Finke, & Van Huis, 2018). Data are now being collected to determine the impacts of feeding a BSFL-based diet on gut and animal health in aquaculture and agricultural settings. Given their favourable nutrient profile and low environmental impact, BSFL are being investigated

as an alternative protein source across several sectors. One of the most active areas of research is their application in aquaculture feed.

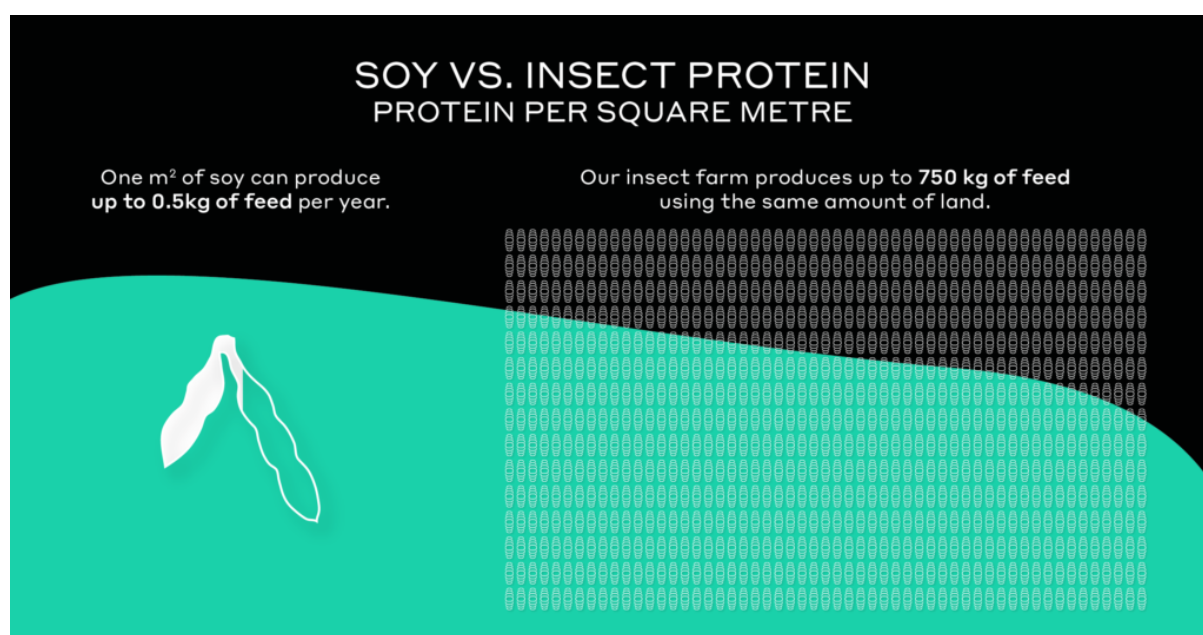


Figure 1.2 Comparison of protein yield per square metre between soy and insect farming. One square metre of soy can produce up to 0.5 kg of feed per year, whereas insect farming can yield up to 750 kg of feed from the same area, highlighting the superior land-use efficiency of insect protein production (Better Origin, 2020).

1.15. BSFL in Aquaculture: A sustainable protein source with functional benefits

Black Soldier Fly Larvae (BSFL) have emerged as a promising alternative to conventional aquafeed ingredients such as fishmeal and soybean meal due to their favourable nutritional profile, environmental advantages, and functional properties. With the aquaculture sector projected to be the fastest-growing food-producing industry globally (FAO, 2022(b)) the need for sustainable and cost-effective feed solutions has never been greater.

Several studies have demonstrated that BSFL can successfully replace fishmeal in the diets of a variety of aquaculture species without compromising growth performance, feed conversion ratio, or health parameters. For instance, Nile tilapia (*Oreochromis niloticus*) fed diets incorporating BSFL meal showed enhanced mucosal immunity through elevated skin mucus lysozyme and peroxidase activities, with no negative

effects on survival, haematology, or growth (Tippayadara, et al., 2021). Similarly, juvenile rainbow trout (*Oncorhynchus mykiss*) displayed no physiological impairments when fed BSFL oil, which was deemed a viable substitute for fish and soybean oils (Fawole F. J., 2021(b)).

Beyond growth and digestibility, several studies have highlighted the immunomodulatory potential of BSFL diets. In juvenile barramundi (*Lates calcarifer*), BSFL inclusion improved immune responses and stress resistance (Caimi, Renna, Sogari, & Gasco, 2021), while in European seabass (*Dicentrarchus labrax*), BSFL meal enhanced antioxidant capacity, immune activity, and survival following challenge with *Vibrio alginolyticus* (Magalhães, et al., 2017).

These effects are likely mediated by functional components inherent to BSFL, including medium-chain fatty acids like lauric acid, chitin, and antimicrobial peptides (AMPs), which may enhance gut health and immune responses in fish (Gasco, Finke, & Van Huis, 2018) (Henry, Gasco, Piccolo, & Fountoulaki, 2022). In fact, BSFL-fed fish have shown increased microbial diversity and beneficial gut bacteria, suggesting a prebiotic effect (Bruni, Pastorelli, Viti, Gasco, & Parisi, 2018). Moreover, BSFL meal has been explored to improve palatability and nutrient digestibility, particularly in carnivorous species (Mastoraki, I. T., & Mente, 2020).

However, optimal inclusion levels vary by species. While some fish tolerate up to 50-75% replacement of fishmeal, others may require more gradual substitution to avoid palatability issues or unbalanced amino acid profiles (Sánchez-Muros, Barroso, & Manzano-Agugliaro, 2014) (Barroso, et al., 2019).

Thus, further studies on species-specific formulations, processing methods, and long-term impacts are warranted to fully unlock the potential of BSFL in aquafeeds.

Taken together, these findings position BSFL not only as a sustainable protein source for aquaculture but also as a functional feed ingredient with promising effects on fish

health, welfare, and disease resistance, aligning well with the industry's growing interest in precision nutrition and reduced antibiotic use.

1.16. Antimicrobial Peptides: *Hermetia illucens*

The Black Soldier Fly has over 50 genes that encode putative AMP expression, which has been reported in only one other insect, the harlequin ladybird *Harmonia axyridis* (Vogel, Mřller, Heckel, Gutzeit, & Vilcinskaskas, 2018) (Vilcinskaskas, Mukherjee, & Vogel, 2013). Furthermore, 26 genes encoding putative defensins were identified, the highest number reported from animals (Vogel, Mřller, Heckel, Gutzeit, & Vilcinskaskas, 2018). Vogel, et al., also found that nutritional components of feed have a significant impact on the expression of immunity-related genes in BSFL. In their study in nutritional immunology, they found that food supplemented with a mixture of bacteria had a significant impact on gene expression and overall antimicrobial activities. After their disease challenge, Park et al. isolated a novel AMP in *Hermetia illucens*, which exhibited activity against Gram-positive bacteria (Park, Kim, & Yoe, 2015). Referred to as defensin-like peptide 4 (DLP4), this AMP showed antibacterial effects against methicillin-resistant *Staphylococcus aureus* (MRSA). Mice had a survival rate of 80-100% between doses 3mg/kg-7.5mg/kg (Li, et al., 2017). Van Moll et al. evaluated the activity of 36 black soldier fly AMPS against a variety of human pathogens and a human cell line (Van Moll, et al., 2022). The cecropin AMPs, Hill-Cec1 and Hill-CEC10, showed no signs of in vitro haemolysis or cytotoxicity in lung fibroblasts and erythrocytes. Furthermore, they were found to be active against *E. coli*, various *P. aeruginosa* species (including a multidrug-resistant strain), and *K. pneumoniae*, exhibiting a fast-killing time. Both AMPs were also capable of significantly reducing biofilm mass when added to planktonic *P. aeruginosa* bacteria (Van Moll, et al., 2022). CLP1 (cecropin-like peptide 1) demonstrated that the inhibition of *E. coli* was 50-fold greater than that produced by ampicillin (Park & Yoe, 2017). The N-terminal primary structure of CLP1 is considered important for antibacterial function. Moretta et al. focused on the gene level to identify all putative genes expressing AMPs (Moretta, et al., 2020). Using a bioinformatic approach, they identified 57 peptides to serve as a starting point for subsequent

functional characterisation of *H. illucens* AMPs produced by recombinant methodologies and chemical synthesis. Ongoing research continues to explore the therapeutic value of these peptides, potentially positioning BSFL as a future source of next-generation antibiotics.

1.17. Barriers to commercialisation and standardisation of BSFL production

While the Black Soldier Fly (*Hermetia illucens*) has gained substantial attention as a sustainable protein source, the industry is still navigating several critical challenges that hinder its widespread commercial adoption. One of the foremost issues is the need for industrial-scale upscaling. Although numerous pilots and small-to-medium enterprises (SMEs) have demonstrated the feasibility of BSFL production, transitioning to a consistent, high-volume output capable of competing with established feed sources remains a complex and capital-intensive undertaking.

A significant barrier to commercial competitiveness is the price of BSFL meal compared to traditional protein sources like fishmeal. Currently, BSFL meal is often priced substantially higher, with costs ranging between £1,500 and £2,500 per metric tonne, while conventional fishmeal averages £1,200 to £1,600 per tonne (Thiermann, 2024) (Global price of Fish Meal, 2025) (prices may vary with region and market volatility). This price gap is largely due to the higher costs of production, particularly energy, labour, and substrate sourcing. Additionally, feedstock availability and cost can fluctuate seasonally, making consistent, low-cost input streams a logistical challenge. Until BSFL can be produced at economies of scale like those seen in the fishmeal and soybean industries, price parity will remain difficult to achieve.

Beyond economics, the sector also faces regulatory and consumer perception hurdles. In many regions, legislation around the use of insect protein, particularly for aquaculture, poultry, and pet feeds, is still evolving. Regulatory uncertainty can dissuade investment and delay product development timelines. Furthermore, despite growing interest in sustainable protein sources, consumer awareness and acceptance

of insects in the food chain (even indirectly, via animal feed) remains relatively low in Western markets.

The nutritional composition of black soldier fly larvae (BSFL) is influenced by factors such as the larvae's diet, developmental stage, and processing methods. Studies have shown that protein content in BSFL can vary significantly across different life stages, with reported values of 31.1% in larvae, 39.0% in pupae, and 65.5% in adults (Barragan-Fonseca & Dicke, 2017). Additionally, the crude protein content has been observed to fluctuate throughout larval development, ranging from 56.2% in one-day-old larvae to 38.0% by the twelfth day (Oonincx, et al., 2010). The larvae's diet also plays a crucial role; for instance, BSFL reared on different organic waste substrates exhibited variations in their nutritional profiles, reflecting the composition of the feedstock (Spranghers, 2017). Furthermore, processing methods can impact the nutritional value, with factors such as drying techniques affecting protein and fat content (van Broekhoven, 2015). These variations underscore the importance of standardising rearing and processing conditions to ensure consistent nutritional quality in BSFL-derived products.

1.18. Global Regulatory Landscape for Insect-based Feed

The regulation of insect farming for feed applications varies significantly across global regions, with some countries actively embracing insect protein as part of circular economy strategies, while others remain cautious due to food safety concerns or legislative gaps. Regulatory acceptance hinges primarily on two key factors: the insect species permitted for use and the substrates on which they can legally be reared (van Huis & Oonincx, 2017)

In the European Union, insect protein has been authorised for use in aquaculture since 2017 and, more recently, in poultry and pig diets following the implementation of Regulation (EU) 2021/1372. This amendment to Regulation (EC) No 999/2001 lifted the ban on processed animal protein (PAP) from insects for monogastric livestock, provided the insect species are among the seven authorised for feed use,

including *Hermetia illucens* and *Tenebrio molitor* (EFSA, 2021). However, insects can only be reared on Category 3 materials, former foodstuffs of non-animal origin or certain animal by-products deemed safe but unsuitable for human consumption, under Regulation (EC) No 1069/2009. The use of catering waste, manure, or meat-containing waste remains prohibited (European Commission, 2009).

In the UK, post-Brexit legislation largely mirrors EU rules, with the Animal By-Products (Enforcement) Regulations 2013 still in force. While the UK has begun industry consultations on expanding approved substrates, no significant legal changes have yet been implemented (FSA, 2023)

In Asia, regulatory approaches are often more permissive or less standardised. In China, BSFL is widely utilised in feed, especially in aquaculture and poultry sectors, though comprehensive national standards are still under development (Liu, et al., 2020). Thailand and Vietnam support BSFL use in feed, with public–private partnerships advancing insect farming for organic waste reduction and protein production (Halloran, Roos, Eilenberg, Cerutti, & Bruun, 2018). In Japan and South Korea, insects are gaining popularity in both pet and aquafeed sectors, and specific hygiene guidelines are gradually being introduced to support regulatory clarity (Nakagaki & Defoliart, 1991).

In the Americas, regulation is relatively progressive in North America but still emerging in Latin America. In the United States, BSFL-derived ingredients have been approved by the Food and Drug Administration (FDA) and Association of American Feed Control Officials (AAFCO) for use in aquaculture, poultry, and pet food (AAFCO, 2023). However, rearing substrates are restricted to pre-consumer food waste unless otherwise authorised. Canada has approved BSFL meal for use in aquaculture, poultry, and swine feed, and is considered one of the more advanced jurisdictions in terms of regulatory guidance and product safety evaluation (CFIA, 2022). In Latin America, countries such as Brazil and Colombia are showing increasing interest in insect farming, but national regulatory frameworks remain limited or fragmented (Gasco, Biancarosa, & Liland, 2020).

In summary, while global momentum for insect-based feed is growing, regulatory disparities remain. The EU and UK are making cautious progress with tight substrate controls. Asian countries show flexibility driven by cultural acceptance and food security priorities, while North America is taking a science-based, incremental approach to approval. Harmonising international regulations and expanding approved substrate lists will be key to scaling the BSFL industry sustainably and unlocking its full potential for waste valorisation and alternative protein production.

1.19. Life Cycle Analysis

A Life Cycle Assessment (LCA) to determine the potential environmental impacts of BSFL-mediated bioconversion showed that, for the functional unit of 1 tonne of food waste treated, it was valued at 30.2kg CO₂ in terms of global warming potential, 215.3 MJ in terms of energy use, and 0.661 m²a in terms of land use. When comparing this against existing waste management technologies, N fertilisers and feed protection, biodiesel, among others, these results were minimal in comparison (Salomone, et al., 2017). A study done by Perdednia et, al. showed that BSFL completely consumed feed in 7 days, compared to 45 days for microbial decomposition. Furthermore, when BSFL were present, 25.54% of carbon initially present within the system was released into the atmosphere. Conversely, when BSFL were absent, 48.62% of the original carbon was lost to the environment (Perednia, Anderson, & Rice, 2017). BSFL can sequester carbon, storing it in the form of proteins, edible oils, and chitin, all having economic value. BSFL emit less ammonia and greenhouse gases and require less land and water than the rearing of other animals (Oonincx, et al., 2010) (Van-Huis, 2013). In this context, these findings suggest that insect-mediated recycling is a sustainable method to recycle organic materials, while producing valuable chains for other commercial purposes.

These life cycle insights not only support the integration of BSFL into waste management systems but also align with global climate targets aimed at reducing carbon emissions and improving resource efficiency. However, further comparative

studies are needed across diverse geographic regions and feedstock types to capture the full environmental footprint under different operational scales.

Additionally, harmonised LCA methodologies are essential to ensure consistency and comparability across insect-based waste treatment models. This will enable policymakers and stakeholders to better quantify the sustainability benefits of BSFL integration into circular bioeconomy frameworks.

1.20. Innate Immunity and Host-Microbiome Interactions in *Hermetia illucens*

The ecological success of the Black Soldier Fly (*Hermetia illucens*) is largely attributed to its ability to thrive within environments characterised by high microbial abundance, intense competition for resources, and continual exposure to potential pathogens. Throughout their larval development, Black Soldier Fly Larvae (BSFL) inhabit decomposing organic matter, including food waste, agricultural residues, manure, and other nutrient-rich substrates undergoing microbial degradation. Such environments contain dense and diverse communities of bacteria, fungi, viruses, and protozoa, creating substantial selective pressures on host defence systems. Consequently, the capacity of BSFL to tolerate, regulate, and exploit these microbial communities is central to both their ecological success and industrial utility.

Understanding the biological mechanisms underpinning this resilience is essential for understanding both the ecology and industrial applications of BSFL. Increasing evidence suggests that the success of *H. illucens* is driven by complex interactions between innate immunity, antimicrobial peptide production, gut microbial communities, nutrient metabolism and environmental conditions. The following sections examine the evolution of innate immunity in *H. illucens*, the mechanisms of host defence, the role of the gut microbiome in maintaining intestinal homeostasis, and the ecological interactions that contribute to efficient waste bioconversion and pathogen suppression.

1.21. Evolution of Innate Immunity in *Hermetia illucens*

Unlike vertebrates, insects lack an adaptive immune system and therefore do not produce antibodies or generate long-term immunological memory through specialised B and T lymphocytes. Instead, insect defence relies upon a highly efficient innate immune system capable of rapidly recognising and responding to invading microorganisms (Lemaitre & Hoffmann, 2007). Innate immunity is evolutionarily ancient and is conserved throughout the animal kingdom, providing immediate protection through the detection of pathogen-associated molecular patterns (PAMPs), including lipopolysaccharides, peptidoglycans, β -glucans, and viral nucleic acids. Recognition of these conserved microbial signatures initiates signalling cascades that ultimately activate cellular and humoral immune responses aimed at limiting pathogen proliferation and maintaining host survival.

Insects possess several complementary defence mechanisms that collectively contribute to immune protection. These include physical barriers such as the cuticle and gut epithelium, cellular responses mediated by circulating haemocytes, and humoral responses involving the synthesis of antimicrobial peptides (AMPs), reactive oxygen species, lysozymes, clotting factors, and melanisation-associated enzymes. Together, these mechanisms provide broad-spectrum protection against a diverse range of microbial threats. Although these systems have been extensively characterised in model insects such as *Drosophila melanogaster*, increasing evidence suggests that *H. illucens* possesses an unusually robust innate immune repertoire, likely reflecting adaptation to its septic ecological niche.

The evolutionary history of *H. illucens* differs substantially from that of many other insects due to its close association with decomposing organic matter throughout larval development. Unlike species that exploit relatively stable food sources, BSFL routinely encounter fluctuating microbial populations, including opportunistic pathogens and environmental contaminants. Such conditions are thought to have favoured the expansion and diversification of immune-related gene families involved in pathogen recognition and antimicrobial defence.

The adaptation of BSFL to microbially rich environments has likely been driven by strong selective pressures favouring rapid pathogen recognition and efficient antimicrobial responses. Comparative genomic and transcriptomic studies have revealed expansions in several immune-related gene families, including those associated with pathogen recognition receptors and antimicrobial peptide production (Vogel, Muller, Heckel, Gutzeit, & Vilcinskas, 2018). Such adaptations are proposed to enhance the ability of BSFL to tolerate fluctuating microbial communities while maintaining developmental performance on heterogeneous waste substrates. Importantly, these evolutionary adaptations appear to extend beyond pathogen defence alone, influencing interactions with resident gut microbiota, nutrient acquisition processes and overall ecological fitness.

These immune responses are mediated through both cellular and humoral defence mechanisms, which together protect against microbial invasion while contributing to the maintenance of physiological homeostasis.

1.22. Cellular and Humoral Defences in Black Soldier Fly Larvae

The innate immune system of insects consists of a coordinated network of cellular and humoral defence mechanisms that provide rapid protection against invading microorganisms. Unlike vertebrates, which rely on both innate and adaptive immune responses, insects depend entirely upon innate defence pathways to detect, contain and eliminate pathogens. These responses involve interactions between specialised immune cells, soluble effector molecules and physical barrier tissues, collectively enabling insects to survive within environments characterised by substantial microbial exposure (Lemaitre & Hoffmann, 2007) (Jens & Reynolds, 2009).

Cellular immunity is primarily mediated by haemocytes, the circulating immune cells of the insect haemolymph. Although haemocyte classification varies among insect taxa, these cells perform several conserved immune functions including phagocytosis,

nodulation, encapsulation and wound repair (Lavine & Strand, 2002). Phagocytosis represents one of the first lines of defence against microbial invasion and involves the recognition, engulfment and intracellular destruction of microorganisms. This mechanism is particularly effective against individual bacterial and fungal cells and has been widely reported across Dipteran insects (Strand, 2008). In addition to phagocytosis, haemocytes contribute to nodulation, a process whereby clusters of microorganisms are aggregated and immobilised within multicellular structures. Nodulation is considered one of the most important cellular defence mechanisms in insects and facilitates the rapid sequestration of large numbers of invading microbes (Satyavathi, Minz, & Nagaraju, 2014). Although many of these defence mechanisms have been characterised in model insects such as *Drosophila melanogaster*, emerging evidence suggests that comparable processes operate within *H. illucens*, contributing to its remarkable tolerance of microbially rich waste substrates (Vogel, Muller, Heckel, Gutzeit, & Vilcinskas, 2018).

When pathogens or foreign bodies are too large to be eliminated through phagocytosis, insects employ encapsulation responses. During encapsulation, multiple haemocytes adhere to the surface of the invading organism, forming multilayered cellular capsules that physically isolate the threat from host tissues (Zdybicka-Barabas, Stączek, Kunat-Budzyńska, & Cytryńska, 2025). This response is particularly important for defence against parasitoids, multicellular parasites and other large foreign materials.

Encapsulation is frequently accompanied by melanisation, a humoral immune process involving the deposition of melanin around invading organisms and damaged tissues. Melanisation is mediated through activation of the prophenoloxidase cascade, which generates cytotoxic intermediates capable of restricting pathogen growth while simultaneously contributing to wound healing and tissue repair (Cerenius & Söderhäll, 2004).

In addition to cellular responses, insects produce a diverse range of humoral immune effectors that circulate throughout the haemolymph and provide systemic antimicrobial protection. These include reactive oxygen species, lysozymes, clotting proteins and antimicrobial peptides (AMPs). Among these, AMPs are widely regarded as the most

important inducible immune effectors in insects due to their broad-spectrum activity against Gram-positive bacteria, Gram-negative bacteria, fungi and certain viruses (Bulet, Stöcklin, & Menin, 2004). Following pathogen recognition, AMP expression can increase rapidly, resulting in the production of peptides capable of disrupting microbial membranes, inhibiting cellular metabolism and limiting pathogen proliferation.

Physical barriers provide an additional layer of protection against microbial invasion. The insect cuticle serves as the first line of defence against environmental pathogens, while the gastrointestinal tract acts as a selectively permeable barrier that regulates interactions between the host and ingested microorganisms (Hegedus, Erlandson, Gillott, & Toprak, 2009). Within the digestive system, the peritrophic matrix and gut epithelium contribute to pathogen exclusion while permitting nutrient absorption and the maintenance of beneficial microbial communities. Together, these cellular, humoral and barrier defences form an integrated immune network that enables insects to tolerate continuous microbial exposure while maintaining physiological homeostasis.

The importance of these defence mechanisms is particularly evident in *Hermetia illucens*, whose larval stages develop within highly contaminated substrates containing dense and diverse microbial populations. The ability of BSFL to survive and proliferate under such conditions suggests the presence of highly effective cellular and humoral immune responses that function in concert with microbial regulation and antimicrobial peptide production. Understanding these defence mechanisms provides an important foundation for examining the genetic diversity of antimicrobial peptides and other immune effectors within *H. illucens*.

1.23. Genetic Diversity and Regulation of Antimicrobial Peptides

Comparative genomic and transcriptomic analyses have identified extensive repertoires of antimicrobial peptides within *H. illucens*, including multiple defensins, attacins, cecropins, dipterocins and other immune effectors, many of which appear to have arisen through gene duplication and subsequent diversification. This expanded immune

repertoire is proposed to contribute to the remarkable resilience of BSFL when reared on highly contaminated substrates and may partially explain their capacity to reduce pathogen burdens during waste bioconversion processes (Vogel, Muller, Heckel, Gutzeit, & Vilcinskis, 2018) (Moretta, et al., 2020).

Antimicrobial peptides (AMPs) represent one of the most important humoral defence mechanisms within the insect innate immune system and constitute a major component of host protection against bacterial, fungal and viral pathogens (Bulet, Hetru, Dimarcq, & Hoffmann, 1999). Although AMPs are widely distributed throughout insects, the size and composition of AMP repertoires vary considerably among taxa, reflecting differences in ecological niche, microbial exposure and evolutionary history. Species that routinely encounter highly diverse microbial communities are often characterised by expanded AMP repertoires, suggesting that ecological pressures can drive the diversification of immune effector genes and contribute to enhanced pathogen resistance.

Among insects, *Hermetia illucens* has emerged as a particularly interesting model for AMP research due to the exceptional diversity of antimicrobial peptides identified within its genome and transcriptome. Early transcriptomic investigations by (Vogel, Muller, Heckel, Gutzeit, & Vilcinskis, 2018) revealed extensive AMP diversity in BSFL and demonstrated that AMP expression was strongly influenced by dietary substrate. Subsequent analyses by (Moretta, et al., 2020) identified 57 putative AMPs belonging to multiple functional families, including defensins, attacins, cecropins, dipterocins, stomoxyn-like peptides and Knottin-like peptides. More recent studies continue to expand the catalogue of BSF AMPs, highlighting the complexity of the species' antimicrobial arsenal and its potential importance for both host defence and biotechnological applications.

The remarkable diversity of AMP families observed within *H. illucens* is thought to have arisen, at least in part, through repeated gene duplication and subsequent functional diversification. Gene duplication provides raw material for evolutionary innovation by generating paralogous genes that may accumulate mutations while retaining

antimicrobial functionality (Crow & Wagner, 2006). Over time, these paralogues can diverge in sequence, regulation and target specificity, allowing hosts to respond to a broader range of microbial threats. Such diversification may be particularly advantageous in BSFL, which develop within decomposing substrates containing highly dynamic and taxonomically diverse microbial communities. The presence of multiple defensin and attacin paralogues identified within BSF transcriptomic datasets supports the hypothesis that gene expansion has contributed to the evolution of a flexible and responsive antimicrobial defence system.

In addition to genetic diversity, AMP expression is tightly regulated and highly responsive to environmental conditions (Lemaitre & Hoffmann, 2007). Numerous studies have demonstrated that AMP production can be influenced by microbial exposure, dietary substrate, developmental stage and immune stimulation. Following recognition of pathogen-associated molecular patterns, signalling pathways such as Toll and IMD activate the transcription of AMP genes, resulting in rapid production of effector molecules capable of limiting pathogen proliferation (Myllymäki, Valanne, & Rämetsä, 2014). In BSFL, substrate-dependent changes in AMP expression have been reported during development, suggesting that immune responses may be shaped not only by pathogen exposure but also by interactions between diet, metabolism and resident microbial communities.

Advances in high-throughput sequencing technologies have significantly improved understanding of AMP diversity and regulation within *H. illucens*. Transcriptomic approaches have facilitated the identification of previously uncharacterised immune effectors, provided insights into AMP family expansions and enabled the investigation of gene expression responses following immune challenge. These findings have established BSFL as a valuable model for studying antimicrobial peptide evolution and function, while also providing a foundation for exploring the molecular mechanisms underpinning immune regulation within this increasingly important industrial insect species (Candian, Meneguz, & Tedeschi, 2023).

1.24. The BSFL Gut Microbiome and Intestinal Homeostasis

In addition to direct pathogen defence, increasing evidence indicates that the immune system of *Hermetia illucens* not only functions in pathogen elimination but also contributes to the regulation of beneficial and commensal microbial communities. Rather than acting solely to remove microorganisms, insect immunity plays an important role in maintaining microbial homeostasis by restricting harmful taxa while permitting the persistence of resident microorganisms that support host physiology. This relationship is particularly important within the digestive tract, where gut microbiota contribute to digestion, nutrient metabolism, detoxification and pathogen exclusion. As a result, the ecological resilience of Black Soldier Fly Larvae (BSFL) is increasingly recognised as a product of integrated interactions between host immunity, gut microbial communities, intestinal physiology and environmental conditions.

The BSFL gut microbiome is highly dynamic and is shaped by diet, larval age, genetic background and gut region. Recent work has shown that bacterial communities differ significantly according to host line, diet composition and developmental stage, indicating that the BSFL microbiome should not be considered a fixed or universally conserved community. (Silvaraju, Zhang, Kittelmann, & Puniamoorthy, 2024) identified diet, genetics and larval age as significant drivers of gut bacterial community structure, while also showing that only a limited number of taxa were consistently detected under strict core microbiome criteria. These findings support the view that BSFL possess a flexible microbiome capable of responding to heterogeneous substrates rather than relying on a single invariant microbial community.

Despite this variability, several bacterial groups are frequently reported in BSFL gut studies. Taxa associated with the genera *Providencia*, *Morganella*, *Enterococcus*, *Klebsiella*, *Proteus*, *Dysgonomonas* and related *Enterococcaceae* have been repeatedly detected, although their relative abundance varies with diet, rearing conditions and analytical approach (Klammsteiner, et al., 2020) (Lin & Shelomi, 2024). Importantly, recent studies caution against overstating the presence of a universal core microbiome, as different prevalence thresholds and environmental conditions can strongly influence

which taxa are defined as “core”. For this reason, the BSFL gut microbiome is best described as a functionally adaptable microbial community, with some recurrent bacterial taxa, rather than as a strictly conserved taxonomic assemblage. This functional flexibility may represent an important adaptive trait, allowing BSFL to exploit a wide range of decomposing substrates while maintaining digestive efficiency and host health.

A key feature of BSFL intestinal homeostasis is the strong regionalisation of the larval midgut. The midgut is divided into anterior, middle and posterior regions, each with distinct physiological functions. (Bonelli, et al., 2019) demonstrated that these regions differ in luminal pH, digestive enzyme activity and epithelial morphology. The anterior, middle and posterior midgut regions exhibit distinct physicochemical environments, with the anterior midgut being mildly acidic, the middle midgut strongly acidic, and the posterior midgut alkaline, thereby creating distinct microenvironments along the digestive tract. Subsequent work by (Bonelli, et al., 2020) reported pronounced pH compartmentalisation within the BSFL midgut, with the anterior midgut remaining mildly acidic (pH 5.8-6.0), the middle midgut strongly acidic (pH 1.8-2.4), and the posterior midgut alkaline (pH 8.3–8.8). Importantly, these regional pH conditions were maintained despite substantial differences in dietary substrate composition, suggesting that BSFL actively regulate gut physicochemical conditions to preserve digestive and homeostatic functions.

This pH gradient is likely to be central to both digestion and microbial regulation. The strongly acidic middle midgut, together with lysozyme activity and antimicrobial proteins, may contribute to the reduction of ingested microorganisms and the structuring of microbial communities along the gut. In contrast, the alkaline posterior midgut supports digestive processes such as protein breakdown by enzymes that function most efficiently under alkaline conditions. As a result, gut pH does not simply reflect digestion; it forms part of a broader intestinal defence and homeostatic system that helps regulate microbial survival, nutrient processing and pathogen exclusion (Bonelli, et al., 2019) (Bonelli, et al., 2020).

The influence of gut region on microbial composition has also been demonstrated directly. (Bruno, et al., 2019) showed that the intestinal microbiota of *H. illucens* larvae is affected by both diet and midgut region, with microbial diversity and bacterial load varying along the digestive tract. This supports the idea that the BSFL gut should not be treated as a single homogeneous microbial environment. Instead, the digestive tract contains distinct ecological niches shaped by pH, enzyme activity, host secretions and substrate-derived microorganisms.

The functional role of the BSFL gut microbiome extends beyond taxonomic composition. Gut-associated microorganisms can contribute to the breakdown of complex organic substrates through microbial enzymes such as proteases, lipases and cellulases, thereby supporting nutrient release and larval growth (Ment & Mishra, 2025). These microbial functions are particularly important because BSFL naturally develop on decomposing organic matter, where nutrients may be chemically complex, variable and embedded within microbial biomass. The microbiome therefore contributes to the transformation of heterogeneous waste streams into larval biomass, linking gut ecology directly to the industrial value of BSFL as a bioconversion species.

Dietary substrate is one of the most important factors influencing both microbiome composition and intestinal function. Substrate macronutrient profile can alter microbial communities, digestive enzyme activity and larval performance. For example, (Bonelli, et al., 2020) showed that larvae reared on different substrates altered digestive enzyme activity and midgut morphology, including changes in proteolytic, amylolytic and lipolytic activity. This indicates that BSFL can physiologically adjust their digestive system in response to substrate composition. These changes are likely to interact with microbial community shifts, as diet affects both the nutrients available to the host and the ecological conditions experienced by gut microorganisms.

The gut microbiome also contributes to pathogen suppression. Resident microorganisms may limit pathogen establishment through nutrient competition, occupation of attachment sites, production of inhibitory metabolites and interactions with host immune effectors. (Elhag, et al., 2022) showed that BSFL reduced zoonotic

pathogens in pig manure and suggested that both host antimicrobial peptides and gut-associated bacteria contributed to this inhibitory effect. Their study demonstrated reductions in coliforms, *Staphylococcus aureus* and *Salmonella* spp. during BSFL conversion, while gut-isolated bacteria showed antagonistic activity against selected pathogens in vitro.

Host immunity is therefore likely to operate alongside gut physiology and microbial competition to maintain intestinal homeostasis. Pattern recognition receptors and downstream immune pathways can detect microbial-associated molecular patterns and regulate antimicrobial peptide production, while gut pH, lysozyme activity, digestive enzymes and the peritrophic matrix contribute additional layers of control. In this way, BSFL intestinal homeostasis is maintained through a balance between tolerance and resistance: the host must tolerate beneficial or functionally important microorganisms while restricting microbial overgrowth and pathogen invasion. This balance is particularly important in BSFL because larvae are continually exposed to dense and diverse microbial communities during feeding.

Overall, the BSFL gut microbiome represents a highly dynamic and functionally important component of host biology. It contributes to digestion, nutrient acquisition, waste conversion, microbial regulation and pathogen suppression, while being shaped by diet, gut physiology, developmental stage and host immune activity. The marked pH regionalisation of the midgut provides an additional mechanism through which *H. illucens* can structure microbial communities and regulate pathogen survival.

Understanding these interactions between gut microbiota, intestinal physiology and innate immunity is therefore essential for explaining the ecological resilience of BSFL and for developing improved strategies for waste bioconversion, pathogen management and industrial insect production.

1.25. Diet-Driven Shifts in the Gut Microbiome

Diet is widely recognised as one of the primary factors influencing gut microbiome composition in Black Soldier Fly Larvae (BSFL). Unlike many insects that utilise relatively specialised food sources, *Hermetia illucens* can develop on a diverse range of organic substrates, including food waste, agricultural by-products, animal manures and industrial side streams. Consequently, the microbial communities encountered by larvae vary substantially between rearing environments, creating strong selective pressures that influence both gut microbiota composition and function.

Numerous studies have demonstrated that substrate composition exerts a significant influence on the diversity and abundance of microorganisms present within the BSFL digestive tract. (Bruno, et al., 2019) reported that diet composition was a major determinant of midgut microbial diversity and that microorganisms present within the feeding substrate contributed directly to the microbial communities observed within different gut regions. Similarly, (Tanga, et al., 2021) demonstrated that organic waste substrates induced substantial shifts in gut bacterial and fungal communities, resulting in the coexistence of conserved, variable and potentially pathogenic microorganisms. These findings indicate that the BSFL gut microbiome remains highly responsive to environmental inputs and continuously adapts to substrate-associated microbial populations.

Recent investigations further support the view that BSFL possess a flexible and adaptive microbiome rather than a rigidly conserved microbial community. (Silvaraju, Zhang, Kittelmann, & Puniamoorthy, 2024) identified diet, host genetics and developmental stage as major drivers of microbiome structure, while (Auger, Bouslama, Deschamps, & Derome, 2023) demonstrated that microbiota composition changes throughout development and differs between larvae reared on plant-based and animal-based substrates. Together, these findings suggest that microbial community composition reflects both environmental conditions and host-associated factors, allowing BSFL to maintain digestive functionality across diverse ecological niches.

Importantly, diet-driven changes are not restricted to taxonomic composition alone. Alterations in substrate chemistry can also influence the metabolic capabilities of the gut microbiome. Meta transcriptomic analyses have revealed that different diets enrich distinct microbial pathways involved in carbohydrate degradation, protein metabolism, lipid utilisation and lignocellulose breakdown. (Kariuki, et al., 2023) demonstrated that lignocellulose-rich substrates such as brewers' spent grain, chicken manure and water hyacinth altered microbial gene expression profiles and carbohydrate-active enzyme activities within the gut microbiome. These functional shifts may enhance the ability of BSFL to degrade complex organic materials and contribute to the remarkable substrate flexibility observed in commercial production systems.

The relationship between diet and microbiome composition has important consequences for larval performance. Substrate-induced microbial shifts have been associated with differences in growth rate, biomass accumulation, nutrient conversion efficiency and waste degradation capacity. Changes in microbial community structure may influence nutrient availability through the production of digestive enzymes, vitamin synthesis, nitrogen recycling and the degradation of otherwise inaccessible organic compounds. Consequently, the microbiome is increasingly viewed as an important mediator between diet composition and host physiology, contributing to the efficient conversion of waste streams into larval biomass (Lin & Shelomi, 2024).

Diet-driven microbial shifts may also influence host immunity and pathogen suppression. Different substrates introduce distinct microbial communities and pathogen burdens, requiring continuous adjustment of both microbial and host regulatory mechanisms. Interactions between substrate-associated microorganisms, resident gut microbiota and host immune responses are likely to influence antimicrobial peptide production, pathogen exclusion and intestinal homeostasis. As discussed previously, maintenance of this balance is essential for preventing microbial dysbiosis while preserving the beneficial functions provided by resident microbial communities.

From an industrial perspective, understanding diet-driven microbiome dynamics has considerable practical significance. The ability to manipulate microbial communities through substrate formulation offers opportunities to improve waste bioconversion efficiency, enhance larval growth and increase resistance to pathogens. Recent reviews have highlighted the potential for microbiome engineering, probiotic supplementation and targeted substrate design as strategies to optimise BSFL production systems. As the insect industry continues to expand, the microbiome is increasingly recognised not simply because of diet, but as a potentially exploitable component of BSFL biology capable of influencing productivity, health and environmental sustainability (Vandeweyer, et al., 2023).

Overall, dietary substrate acts as a major driver of gut microbiome structure and function in *H. illucens*. Through its effects on microbial composition, metabolic activity and host-microbe interactions, diet influences nutrient processing, larval performance, pathogen suppression and waste conversion efficiency. Understanding these diet-microbiome relationships is therefore essential for explaining the ecological adaptability of BSFL and for developing more efficient and sustainable insect production systems.

1.26. Linking Immunity, Metabolism and Waste Bioconversion

The preceding sections demonstrate that immunity, microbiome dynamics and nutrient acquisition are closely interconnected within BSFL biology. The maintenance of such an extensive immune system, however, is not without cost. Immune responses are energetically demanding and require the allocation of nutrients that could otherwise support growth, development, reproduction, or nutrient storage (McKean, Yourth, Lazzaro, & Clark, 2008). Consequently, insects must continuously balance investment in immunity against other physiological processes. This phenomenon, commonly described as an immune-metabolic trade-off, is particularly relevant in BSFL due to their rapid growth rates and exceptional capacity for nutrient assimilation. Resources invested in AMP synthesis, melanisation, tissue repair, and immune signalling may

reduce resources available for biomass accumulation, whereas excessive investment in growth could compromise pathogen resistance. Therefore, successful adaptation to decomposing substrates likely depends upon the ability of BSFL to dynamically regulate immune activity according to environmental conditions and microbial exposure.

In ecological immunology, immune function is generally understood as a life-history trait that must be balanced against other fitness-related processes. The costs of immunity can arise from both the maintenance of immune surveillance and the deployment of immune responses following infection or microbial challenge. In resource-limited environments, these costs may become more pronounced because energy and nutrients allocated to defence cannot simultaneously be invested in growth, storage, development or reproduction (Schwenke, Lazzaro, & Wolfner, 2016). Although much of this work has been demonstrated in model insects such as *Drosophila melanogaster*, the concept is highly relevant to *H. illucens* because larvae develop in microbially dense substrates while also undergoing rapid biomass accumulation.

In BSFL, this balance is particularly important because immune competence and bioconversion performance are likely to be interconnected. Larvae must tolerate and regulate dense microbial communities while continuing to digest complex organic substrates and convert nutrients into protein- and lipid-rich biomass. Immune activation may therefore represent both a protective mechanism and a potential metabolic burden (Bruno, et al., 2021). Humoral responses such as AMP production, melanisation and reactive oxygen species generation contribute to pathogen suppression, but their activation requires amino acids, energy and regulatory coordination. Similarly, cellular responses including phagocytosis, nodulation and tissue repair require haemocyte activity and physiological investment. These processes may be essential for survival in contaminated substrates, but they may also influence the efficiency with which nutrients are converted into larval biomass.

Diet and substrate composition are therefore likely to shape not only the gut microbiome but also the balance between metabolism and immunity. Studies on *H. illucens* have shown that dietary substrate can influence AMP expression and

antimicrobial activity, suggesting that immune responses may be modulated by nutritional and environmental conditions (Candian, Meneguz, & Tedeschi, 2023). This is important because organic waste streams differ substantially in protein, carbohydrate, lipid, fibre and microbial content. Nutrient-rich substrates may provide sufficient resources to support both growth and immune activation, whereas nutritionally imbalanced or highly contaminated substrates may increase the physiological cost of maintaining immune protection. As a result, substrate quality may influence whether immune activation supports larval resilience or becomes a constraint on growth and conversion efficiency.

The gut microbiome may help mediate this trade-off by improving nutrient accessibility and contributing to digestive efficiency. Microbial degradation of complex substrates can release nutrients that are otherwise difficult for the host to access, potentially supporting larval growth while also helping to offset the energetic costs of immune activity. At the same time, resident microorganisms can contribute to pathogen exclusion through competition, antimicrobial metabolite production and interactions with host immune pathways. In this way, the microbiome may reduce the need for excessive immune activation by stabilising the gut environment and limiting pathogen overgrowth. However, when microbial communities become dysregulated or pathogenic taxa dominate, increased immune activity may be required to restore intestinal homeostasis.

These interactions have direct implications for BSFL waste bioconversion systems. Industrial production aims to maximise larval growth, survival, biomass yield and waste reduction efficiency, but these outcomes depend upon the ability of larvae to maintain health while processing heterogeneous and microbially active substrates. If immune activation is excessive, resources may be diverted away from biomass accumulation; if immune activity is insufficient, larvae may become more vulnerable to pathogen proliferation and reduced performance. Optimising BSFL production therefore requires understanding not only the nutritional composition of substrates, but also how substrate-associated microbial communities influence immune activation, gut homeostasis and metabolic efficiency (Bruno, et al., 2026).

Overall, the relationship between immunity, metabolism and waste bioconversion highlights the importance of viewing BSFL as an integrated host-microbe system. Their success on decomposing substrates is not simply the result of a strong immune system or efficient digestion alone, but rather the coordinated regulation of microbial exposure, immune defence, nutrient acquisition and growth. Understanding these trade-offs provides an important framework for improving BSFL production systems, particularly through substrate formulation, microbiome management and strategies that support larval health without compromising bioconversion efficiency.

1.27. Eco-Evolutionary Interactions Between BSFL, Waste Streams and Microbial Communities

Understanding these interactions is particularly important given the growing industrial use of BSFL in waste management, feed production and bioconversion systems. The ability of BSFL to process diverse organic waste streams while simultaneously reducing pathogen loads suggests that immune function, microbial ecology and nutrient metabolism are intrinsically linked (Vogel, Muller, Heckel, Gutzeit, & Vilcinskis, 2018). Consequently, examining BSFL through the interconnected lenses of immunity, microbiome dynamics and ecological adaptation provides an important framework for understanding both their biological success and their value as a sustainable biotechnological platform.

Organic waste substrates represent highly dynamic ecological environments characterised by fluctuating nutrient availability, diverse microbial communities and variable pathogen burdens. Unlike insects that exploit relatively stable food sources, BSFL develop within habitats that expose them to continual microbial challenge while simultaneously requiring efficient nutrient acquisition and rapid growth (Ment & Mishra, 2025). As a result, larvae experience concurrent selective pressures associated with digestion, microbial regulation and pathogen defence. The ability of *H. illucens* to thrive under these conditions suggests that immune function, gut physiology and microbial interactions have co-evolved to support survival within decomposing organic matter.

Over evolutionary timescales, adaptation to microbially rich environments has likely contributed to the diversification of antimicrobial peptides, expansion of immune-related gene families and development of physiological mechanisms that facilitate microbial regulation while maintaining digestive efficiency. These adaptations extend beyond pathogen defence alone and influence interactions with resident microbiota, nutrient processing and overall host fitness. Consequently, the adaptive success of BSFL appears to reflect the integrated effects of host immunity, microbiome dynamics and metabolic flexibility rather than any single biological trait.

Importantly, these eco-evolutionary interactions also underpin many of the characteristics that make BSFL valuable for industrial applications (Lin & Shelomi, 2024). Waste conversion efficiency, pathogen suppression and biomass production emerge from the coordinated activity of larval metabolism, resident microbial communities and innate immune defences. During waste bioconversion, substrate-associated microorganisms interact with the larval gut environment, where digestive processes, antimicrobial peptides and microbial competition collectively influence community structure and function (Tanga, et al., 2021). The resulting interactions contribute not only to nutrient acquisition and larval growth, but also to reductions in microbial pathogens and improved substrate stabilisation.

Understanding these relationships has important implications for the future development of BSFL production systems. Improved knowledge of host-microbiome interactions, immune regulation and substrate-associated microbial dynamics may facilitate the optimisation of waste conversion processes, microbiome engineering strategies and pathogen management approaches. Such insights could ultimately improve production efficiency while supporting the safe and sustainable utilisation of BSFL in circular bioeconomy systems.

Overall, the ecological resilience and industrial utility of *H. illucens* arise from the integration of immune defence, digestive plasticity, microbiome dynamics and nutrient conversion. Viewing BSFL as an interconnected host-microbe system provides an

important conceptual framework for understanding how evolutionary adaptation to decomposing organic matter has generated one of the most effective biological platforms currently available for sustainable waste bioconversion and resource recovery.

1.28. Translating BSFL Immunobiology into Industrial Applications

The growing commercialisation of Black Soldier Fly Larvae (BSFL) has increased demand for a more comprehensive understanding of the biological mechanisms underpinning larval health, productivity and disease resilience. As industrial production systems continue to scale, challenges associated with pathogen management, variable substrate quality and production efficiency are becoming increasingly important. Consequently, knowledge of BSFL immunity, microbiome dynamics and host-microbe interactions has significant potential to inform the development of novel technologies and optimise existing production practices.

One promising application lies in the optimisation of rearing substrates through improved understanding of diet-microbiome interactions. As discussed previously, dietary composition influences gut microbial community structure, digestive function and immune activity (Lin & Shelomi, 2024). This knowledge may facilitate the design of substrates that selectively promote beneficial microbial communities while suppressing undesirable microorganisms. Such approaches could improve nutrient utilisation, enhance larval growth and increase waste conversion efficiency. Furthermore, understanding how specific substrates influence microbial and immune responses may help producers identify feedstocks that maximise both productivity and larval health.

The increasing recognition of the gut microbiome as a key determinant of BSFL performance has also generated interest in microbiome engineering approaches. Like the use of probiotics and prebiotics in livestock production, targeted manipulation of

BSFL-associated microbial communities may offer opportunities to enhance nutrient acquisition, improve pathogen resistance and stabilise production systems (Ment & Mishra, 2025). The identification of microbial taxa associated with efficient waste conversion or disease suppression may facilitate the development of microbial supplements capable of improving larval performance under commercial conditions. Although such approaches remain in their infancy, advances in microbiome research are likely to accelerate their development and application within industrial insect farming.

Improved understanding of immune regulation may also support the development of diagnostic and health-monitoring tools for BSFL production systems. The identification of immune biomarkers, including antimicrobial peptides (AMPs), pathogen recognition receptors (PRRs) and other immune-responsive genes, may enable the development of molecular indicators of larval health and environmental stress (Candian, Meneguz, & Tedeschi, 2023). Such biomarkers could provide early warning systems for disease outbreaks, suboptimal rearing conditions or microbial dysbiosis, allowing producers to implement corrective interventions before substantial productivity losses occur. The increasing accessibility of molecular technologies may facilitate the integration of such monitoring approaches into future insect production systems.

Recent advances in genomics and transcriptomics further provide opportunities for selective breeding and strain improvement. Characterisation of immune-related gene families, AMP repertoires and host-microbiome interactions may support the identification of genetic markers associated with disease resilience, growth performance and substrate utilisation efficiency (Vogel, Muller, Heckel, Gutzeit, & Vilcinskas, 2018). These resources could contribute to breeding programmes aimed at developing BSFL strains better adapted to specific industrial applications or environmental conditions. In parallel, transcriptomic datasets may facilitate the construction of curated immune gene databases that support future research into insect health and disease management.

The potential applications of BSFL immunobiology extend beyond insect production itself. The diverse repertoire of antimicrobial peptides identified within *H. illucens* has attracted growing interest as a source of novel antimicrobial compounds. Given the increasing global challenge of antimicrobial resistance, insect-derived AMPs have been proposed as potential alternatives or complements to conventional antibiotics in medical, veterinary and agricultural settings. Similarly, understanding the mechanisms through which BSFL suppress pathogens during waste conversion may provide insights into new approaches for biological waste treatment and microbial risk reduction.

Collectively, these advances highlight the importance of integrating immunology, microbiome research and industrial production science. Rather than viewing immunity solely as a mechanism of pathogen defence, emerging evidence suggests that immune regulation plays a central role in shaping microbial communities, maintaining intestinal homeostasis and influencing nutrient utilisation. Continued investigation of these interactions is therefore likely to generate novel technologies capable of improving disease resilience, production efficiency and sustainability within the rapidly expanding BSFL industry. Ultimately, translating fundamental knowledge of BSFL immunobiology into practical applications may contribute to the development of more robust insect production systems and support the wider adoption of BSFL within circular bioeconomy frameworks.

1.29. Thesis Aims, Research Questions and Objectives

Overall Thesis Aim:

To investigate the innate immune system of the Black Soldier Fly (*Hermetia illucens*) and evaluate how improved understanding of immune function can contribute to disease management, pathogen control and the optimisation of industrial Black Soldier Fly production systems.

Research Questions	Objective	Chapter
How can reliable experimental methodologies be established for studying immune responses in <i>H. illucens</i> ?	Optimise larval challenge procedures, handling methods and RNA extraction methodologies suitable for downstream molecular analyses.	Chapter 2
Which genes and pathways are present within <i>H. illucens</i> and how can they be characterised?	Identify and characterise genes and pathways in <i>H. illucens</i> using transcriptomic analyses, protein interaction networks and pathway enrichment analyses.	Chapter 3
How do selected antimicrobial peptides (AMPs) and pathogen recognition receptors (PRRs) respond to immune stimulation?	Characterise AMP and PRR gene expression responses following bacterial and viral mimic challenge using quantitative PCR. Perform <i>In silico</i> analyses to better understand structure of targets.	Chapter 4

Evaluate the efficacy of prophylactic and remedial phage treatments against <i>S. Typhimurium</i> infection.	Build a challenge model and determine the efficacy of prophylactic and remedial bacteriophage treatment for controlling <i>S. Typhimurium</i> in <i>H. illucens</i> larvae.	Chapter 5
How can improved understanding of the innate immune system of <i>H. illucens</i> contribute to disease management, pathogen control and optimisation of industrial Black Soldier Fly production systems?	Integrate findings from immune challenge, transcriptomic and phage therapy studies to identify opportunities for disease management and sustainable insect production.	Thesis-wide objective

1.30. Thesis Outputs and Key Findings

This thesis investigated the innate immune system of *Hermetia illucens* and explored its application for disease control and sustainable insect production. The principal outputs of this research were:

- Established and optimised experimental methodologies for immune research in BSFL, including larval handling, immune challenge procedures, and whole-body RNA extraction protocols suitable for downstream molecular analyses.
- Generated and curated a transcriptome-derived immune gene resource for *Hermetia illucens*, identifying canonical components of the Toll, IMD, JNK, and JAK/STAT pathways and providing a framework for future functional immunological studies.
- Demonstrated that immune stimulation with LPS and Poly(I:C) induces measurable changes in antimicrobial peptide and pathogen-recognition receptor expression, confirming the suitability of these PAMPs for investigating innate immune responses in BSFL.
- Validated key antimicrobial peptide and immune-related gene targets, including defensins, attacins, cecropin, and Toll-like receptor 6, providing molecular markers for future host-pathogen and immune challenge studies.
- Provided proof-of-concept evidence that prophylactic bacteriophage treatment can reduce *Salmonella enterica* serovar Typhimurium burden in BSFL, supporting the potential application of phage-based disease management strategies within commercial insect production systems.

Chapter 2. Methodology and Optimisation of Molecular Techniques: *Hermetia illucens* (The Black Soldier Fly)

2.1. Introduction

The development of robust and reproducible methodologies is essential for advancing non-model organisms as experimental systems and for ensuring that biological observations can be interpreted with confidence. While *Hermetia illucens* has received considerable attention for its applications in waste bioconversion, sustainable feed production, and biotechnology, relatively few studies have focused on establishing standardised molecular and immunological research methodologies. Consequently, differences in larval rearing conditions, developmental stage, injection procedures, immune challenge protocols, and RNA extraction methods can introduce substantial experimental variation and limit comparisons between studies.

The overarching aim of this chapter was to optimise key methodological parameters required for the subsequent investigation of innate immune responses and host–pathogen interactions in *H. illucens*. Attention was given to variables expected to influence downstream transcriptomic and gene expression analyses, including rearing temperature, larval weight, immune stimulation procedures, sample preservation, and RNA extraction quality. Establishing reliable experimental protocols was considered essential to ensure that any observed biological responses reflected genuine immune activation rather than methodological artefacts.

The optimised approaches described in this chapter formed the foundation for all subsequent experimental work presented in this thesis, including transcriptome generation, antimicrobial peptide expression profiling, and bacteriophage-based disease control studies.

2.2. The Use of Insects in Scientific Research

Insects have long served as powerful model organisms in biological research, offering unique advantages for studying development, immunity, genetics, and host-pathogen interactions. Their short generation times, ease of maintenance, high fecundity, and conservation of key molecular pathways make them ideal for both fundamental and applied scientific inquiry.

One of the earliest examples of biologically derived therapeutics from invertebrates comes not from insects but from annelids. Hirudin, a potent thrombin inhibitor found in the salivary glands of the medicinal leech (*Hirudo medicinalis*), was first discovered to possess anticoagulant properties in 1883 by Berry Haycraft (Haycraft, 1883), then later isolated by Karl Jacoby in 1904 (Jacoby, 1904), paving the way for the development of further anticoagulant therapies, including heparin (George, 1998).

Among insect models, the fruit fly *Drosophila melanogaster* has been particularly instrumental in advancing our understanding of genetics, development, and disease. Due to the evolutionary conservation of many signalling pathways between flies and vertebrates, *Drosophila* has become a central tool in studying oncogenic processes. Research has shown that key pathways involved in tumour formation and progression, including Ras/MAPK, PI3K/Akt, and Notch, are conserved and functionally testable in *Drosophila*, making it a robust model for cancer biology (Rudrapatna, 2012).

Additionally, *Drosophila* models have contributed significantly to the discovery and functional validation of antimicrobial peptides (AMPs), immune signalling cascades (e.g., Toll and IMD pathways), and stress responses (Lemaitre & Hoffmann, The host defense of *Drosophila melanogaster*., 2007) (Buchon, Silverman, & Cherry, 2014).

Beyond model species, interest in non-traditional insects has grown significantly, particularly with the rise of insect farming industries. One such species is the Black Soldier Fly (*Hermetia illucens*), valued not only for its waste bioconversion capabilities and high-protein larvae but increasingly as a subject of immunological and microbiological research. *Hermetia illucens* larvae express a diverse range of AMPs,

including defensins, cecropins, and attacins, which are upregulated in response to microbial challenge (Vogel H. , Müller, Heckel, Gutzeit, & Vilcinskis, 2018). These AMPs show potential as novel anti-infective agents, especially in the face of rising antimicrobial resistance, and have sparked interest in understanding BSFL innate immune mechanisms more comprehensively.

As *Hermetia illucens* continues to gain attention for its applications in food security, waste bioconversion, and circular bioeconomy, there is a growing need to establish optimised, reproducible research methodologies that enable high-quality immunological and molecular studies. Despite the species' growing importance, protocols for experimental immune challenge, RNA extraction, and gene expression analysis remain poorly defined, especially when compared to established model insects such as *Drosophila melanogaster* and *Galleria mellonella*.

This methodological gap presents a barrier to high-throughput studies on BSFL immune function, transcriptomic profiling, and downstream molecular applications. Standard TRIzol protocols, column-based kits, and injection approaches often perform sub-optimally when applied to BSFL due to their high lipid content, chitinous cuticle, and unique physiological traits.

This chapter, therefore, describes a series of methodological innovations developed and optimised specifically for BSFL. These include protocols for larval immune stimulation using pathogen-associated molecular patterns (PAMPs), improved RNA extraction and quality control workflows. These optimisations were undertaken to establish a reliable experimental platform for the transcriptomic analyses described in Chapter 3, the qPCR-based immune profiling presented in Chapter 4, and the bacteriophage challenge studies reported in Chapter 5.

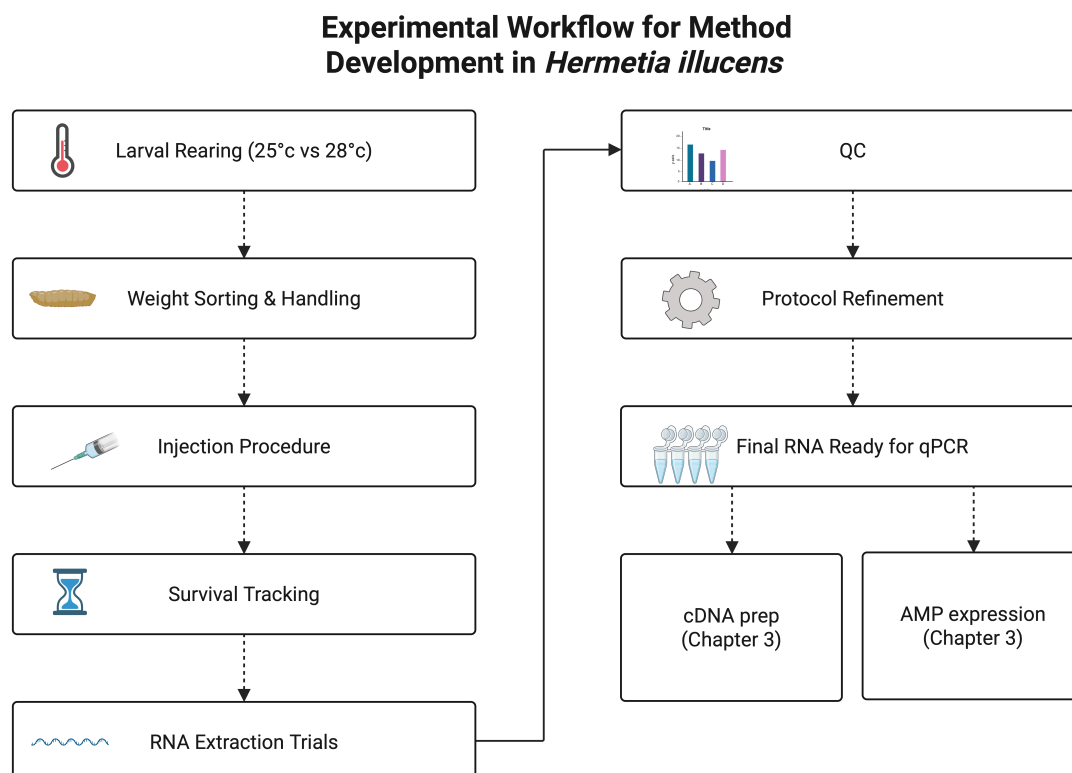


Figure 2.1 An overview of the methodological workflow summarising the sequential stages from larval rearing through to RNA extraction and downstream molecular applications

2.3. Materials and Methods

2.4. *H. illucens* colony maintenance

Black Soldier Fly Larvae were obtained from Better Origin (Cambridge, UK). BSFL were reared at commercially relevant conditions: $29 \pm 0.8^\circ\text{C}$ and 70% relative humidity. Larvae were fed Better Origin's BSF Feed mixed 30:70 with non-sterile H_2O under the same conditions. Strain Origin was used to optimise for downstream analysis.

2.5. Transportation of Larvae

Sistema KLIP IT containers (64.2oz/1.9L Phthalate & BPA free) were used to store and transport larvae to The University of Stirling using Royal Mail's Special Delivery service, which is guaranteed by 1 pm the following day. A hole was made in the lid and covered

with a breathable mesh membrane to circulate air flow, and heat pads were placed in the box to maintain heat during transport. 12DOL (day-old-larvae) arrived with extra feed to rear them on until experimental procedures were complete.

2.6. Sorting larval weight

Larvae were sorted into large ($200\text{mg} \pm 20$) and small ($100\text{mg} \pm 20$) using a multi-purpose portable balance (NVE series, OHAUS). Larvae were placed onto a tray, weighed and sorted into their relevant weight class; they were then stored in containers with feed.

2.7. Determining life cycle changes when rearing larvae at 28°C and 25°C

Optimal rearing conditions for BSFL have been identified within a temperature range of 25-30°C from recent research. Since the larvae have been reared on-site at $29 \pm 0.8^\circ\text{C}$, 70% relative humidity, their life cycle was monitored to confirm that storage at lower temperatures would not adversely affect larval health before PAMP (pathogen-associated molecular pattern) challenge.

For this observation, 12 day-old-larvae (12DOL) ($n=10$ per group) were placed in individual Petri dishes with food and stored inside a Sistema KLIP IT container to maintain consistent humidity and temperature. The larvae were subsequently maintained at $25 \pm 0.5^\circ\text{C}$ and $28 \pm 0.5^\circ\text{C}$, and the number of days required to progress to each subsequent developmental stage (prepupal and pupal phase) was recorded.

Larvae were monitored daily to document the prepupal phase (onset of morphological changes and cessation of feeding) and the pupal phase (formation of a fully developed puparium).

The mean time ($\pm$ standard deviation) to reach prepupal and pupal phases was calculated for each temperature condition. Before performing statistical tests,

Levene's test was conducted to check for equal variance. A two-tailed independent t-test was used to compare developmental timing between temperature groups.

2.8. Optimising Injection Procedure in BSFL

Limited injection-based studies have been conducted using *Hermetia illucens* larvae (BSFL), necessitating optimisation of the injection protocol to ensure high survival rates for downstream analysis. Larvae were delivered at 12 days old (DOL) and immediately placed into a 25°C incubator. Individuals were sorted into groups of 200mg ± 20mg for standardisation, and before injection, larvae were anaesthetised on ice for 1 minute to minimise movement and stress. All experiments were carried out with AWERB 2023 12442 11402.

Survival rates were tracked at 24-, 48-, and 72-hours post-injection for three treatment groups: 10 µl of Saline (0.85% NaCl), PBS (Phosphate-buffered Saline solution), and an injection-only control (no volume) using a Hamilton syringe. The injection site was between the third last and penultimate metamere, allowing systemic distribution via the haemolymph. Data were recorded as a percentage of surviving larvae per group (n=75 per condition). A survival trend graph was generated using Python's Matplotlib library, plotting survival rates over time for each treatment group.

To assess differences in survival across treatment groups, a Chi-Square test for independence was performed at 24-, 48-, and 72-hours post-injection. This test was used to determine whether the injection solution (Saline, PBS, or Injection-Only) had a significant effect on larval survival. Survival data were analysed as categorical variables (alive vs. dead), and p-values were used to evaluate statistical significance. All analyses were conducted using Python's step function (step ()) from matplotlib (version 3.10.0), and pandas (version 2.2.3) for structuring.

2.9. Immune stimulation in BSFL: Determining survival % for 100mg and 200mg Black Soldier Fly Larvae after injection of Lipopolysaccharide (LPS) and Polyinosinic:polycytidylic acid (Poly (I:C))

In this study, four experimental groups were established: Control (no treatment), phosphate-buffered saline (PBS) injection group, an injection-only group (no volume), and treatment groups LPS and Poly (I:C) at doses of 2 µg, 200 ng, 20 ng, and 2 ng, with all injection volumes at 10µl. For each treatment group and concentration, 10 larvae were used per technical replicate, with three technical replicates per biological replicate (30 larvae per treatment group and concentration). The experiment was independently repeated across three biological replicates, giving a total of 90 larvae per treatment group and concentration. This process was completed on larvae from two different weight classes: $100 \pm 20\text{mg}$ and $200 \pm 20\text{mg}$, and their survival was monitored at 0-, 12-, and 24-hour post-treatment when stored at 25°C. Larvae were considered alive if they exhibited movement when gently stimulated with a soft probe. All analyses were executed using a Python script, using a step function (step()) from matplotlib (version 3.10.0) and pandas (version 2.2.3) for structuring. A colour-blind-friendly palette (Okabe-Ito) was applied, and slight offsets were introduced to prevent overlapping survival curves.

2.10. Sample capture and long-term storage of larvae

After 24-, 48-, and 72-hour survival was recorded, larvae were subsequently flash frozen in liquid nitrogen and stored at -80°C to preserve RNA for downstream analysis.

2.11. Extracting RNA from whole-body larvae following TRIZOL manufacturer's protocol

Larvae (whole-body) were placed in 1ml of TRIzol for 1 hour and homogenised in a bead beater for 1 minute. The remainder of the extraction followed the TRIzol method according to manufactures instruction.

2.12. Optimisation of whole-body RNA extraction in *Hermetia illucens*

RNA extraction from BSFL posed significant challenges, likely due to their high fat content, which complicated downstream processing. The standard TRIzol extraction protocol was not directly applicable due to excessive phenol carryover, necessitating protocol optimisation to yield high-quality RNA suitable for further analysis. To improve the extraction process, several modifications were tested, including varying larval sizes, incubation times in TRI reagent, experimenting with different homogenization techniques, and different phase separation reagents by testing different volumes of BCP and chloroform. In addition, RNeasy mini kits were tested to compare against TRIzol extractions.

RNA precipitation was evaluated both with and without the addition of RNA precipitation solution, and the optimal number of ethanol washes was determined to ensure high-quality RNA. A key issue observed during the process was the persistence of residual RNA pellets after resuspension. RNA integrity and concentration were assessed before and after heating the sample to dissolve any remaining pellet. All A260/230 and A260/280 ratios were recorded on a Thermo Scientific Nanodrop 2000c.

2.13. Optimised RNA extraction method

Total RNA was extracted from larvae (n=6) from each treatment group across all concentrations using an optimised TRIzol protocol. Due to the protein and lipid profile of the larvae, each larva was dissected into 3 sections (longitudinally), extracted, and the total RNA was pooled at the end.

Homogenisation: Each dissected section was placed into a labelled screw-cape tube containing 1mL of TRI Reagent. Samples were incubated on ice for 30 minutes before homogenization using a bead beater for 30 seconds, then incubated on ice for 5 minutes.

Phase Separation: Samples were then centrifuged at 20,000 x g for 15 minutes 4°C, after which 600 µL of each sample was transferred to a new tube with 100 µL of BCP (avoiding any lipid layer upon transfer) and incubated on ice for 15 minutes. After another centrifugation step at 20,000 x g for 15 minutes at 4°C, 200 µL of the aqueous phase was transferred to a fresh tube, taking care not to disturb the interphase.

RNA precipitation: 200 µL of 100% isopropanol (1:1) was added to the aqueous phase, followed by gentle inversion (4-6 times) and incubation on ice for 10 minutes at 4°C. Samples were centrifuged at 10,000 x g for 10 minutes at 4°C, forming a gel-like RNA pellet. The pellet was washed three times with 1 mL of 75% ethanol, flicked to lift the pellet, and centrifuged at 20,000 x g for 5 minutes at room temperature. Residual ethanol was removed by air-drying, and RNA was resuspended in 100 µL of Milli-Q water and incubated at room temperature for 30 minutes to facilitate resuspension. Samples were then subsequently heated at 65°C for 5 minutes to support pellet resuspension without any impacts on RNA integrity,

Long Term Storage: 50 µL of RNA from each larval section was pooled into one tube and kept in -80°C for further downstream analysis.

2.14. Results

2.15. Survival data at 25°C and 28°C: following pupation

The developmental times observed at 25 °C and 28 °C are fully consistent with broader BSFL literature, and they highlight both the thermal sensitivity and the practical trade-offs inherent when choosing rearing temperature. Rearing temperature had a significant impact on the developmental timing of *Hermetia illucens* larvae (Table 2.1-2.4). At 25°C, larvae required an average of 7.0 ± 1.15 days (from 12 days of age) to reach the prepupal stage, and 13.5 ± 1.35 days to reach the pupal stage.

In contrast, larvae maintained at 28°C reached the prepupal stage in just 4.1 ± 1.10 days and pupated within 7.9 ± 0.74 days. A two-tailed independent t-test confirmed that these differences were highly significant for both stages (prepupal: $t = 5.75$, $p = 1.89$

$\times 10^{-5}$, pupal: $t = 11.48$, $p = 1.02 \times 10^{-9}$). These results indicate an approximate 41% acceleration in development at 28°C relative to 25°C, consistent with the temperature-dependent growth patterns previously reported in BSFL.

Similar thermal trends have been widely reported across BSFL studies. Chia et al. demonstrated that larvae reared at 30°C progressed through developmental stages markedly faster than those at 25°C, with a clear linear reduction in duration as temperature increased from 20 to 30°C (Chia S. , et al., 2018). Wang et al. observed comparable acceleration across a broad range of rearing temperatures (from 19 to 34°C), noting that each 2-3°C increment resulted in significantly reduced larval duration.

While both rearing temperatures resulted in consistent developmental trajectories overall, some differences in variability between the two groups were observed. Standard deviation values for prepupal transition were comparable (± 1.15 days at 25 °C vs ± 1.10 days at 28 °C), indicating similar levels of synchronisation at this early stage. However, pupal development at 25 °C showed notably greater variability (± 1.35 days vs ± 0.74 days at 28 °C), suggesting increased heterogeneity in the timing of metamorphosis under cooler conditions.

This broader spread in pupation timing at 25 °C, although modest, proved advantageous for this study's experimental aims. Specifically, rearing larvae at 25 °C reduced the risk of premature pupation, allowing a larger proportion of individuals to remain in the target larval stage during experimental windows. This ensured greater consistency in larval condition during pathogen-associated molecular pattern (PAMP) challenges and improved the reliability of health tracking and post-injection assessments. In contrast, rearing at 28 °C, while promoting faster development and reduced variability, would likely compress the intervention window and increase the risk of losing larvae to pupation before or during immune stimulation trials.

In summary, although rearing at 28°C may be ideal for high-throughput or production-focused applications, 25°C offers a more flexible and manageable developmental

window for immune-focused experimental work. The slightly extended and more variable pupation timing at this temperature supports the methodological decision to conduct PAMP challenges under these conditions, allowing for greater larval viability and synchronisation across replicates.

Table 2.1 Pupation development after the arrival of 12DOL (day-old-larvae) at 25°C

25° c

Larvae ID	Start Age (12DOL)	Days from 12DOL to prepupal phase	Age at prepupal phase	Days from 12DOL to pupal phase	Age at pupal phase
L1	12DOL	7	19DOL	12	24DOL
L2	12DOL	6	18DOL	13	25DOL
L3	12DOL	6	18DOL	14	26DOL
L4	12DOL	8	20DOL	16	28DOL
L5	12DOL	7	19DOL	14	26DOL
L6	12DOL	5	17DOL	15	27DOL
L7	12DOL	8	20DOL	14	26DOL
L8	12DOL	9	21DOL	13	25DOL
L9	12DOL	7	19DOL	12	24DOL
L10	12DOL	7	19DOL	12	24DOL

Table 2.2 Pupation development after the arrival of 12DOL (day-old-larvae) at 28°C

28° c

Larvae ID	Start Age (12DOL)	Days from 12DOL to prepupal phase	Age at prepupal phase	Days from 12DOL to pupal phase	Age at pupal phase
L1	12DOL	4	16DOL	8	20DOL
L2	12DOL	3	15DOL	7	19DOL
L3	12DOL	3	15DOL	8	20DOL
L4	12DOL	3	15DOL	7	19DOL
L5	12DOL	5	17DOL	7	19DOL
L6	12DOL	4	16DOL	9	21DOL
L7	12DOL	3	15DOL	8	20DOL
L8	12DOL	6	18DOL	9	21DOL
L9	12DOL	5	17DOL	8	20DOL
L10	12DOL	5	17DOL	8	20DOL

Table 2.3 Comparisons of pupation time when larvae are reared at 25°C and 28°C, including standard deviation and t-test results

Temperature	Mean Days to Prepupal Phase (± SD)	Mean Days to Pupal Phase (± SD)
25°C	7.0 ± 1.15	13.5 ± 1.35
28°C	4.1 ± 1.1	7.9 ± 0.74

Table 2.4 T-test results comparing pupation at 25°C and 28°C

Comparison	T-Statistic	P-Value
Prepupal Phase (25°C vs 28°C)	5.75	1.89E-05
Pupal Phase (25°C vs 28°C)	11.48	1.02E-09

2.16. Impact of Injection Suspension Medium on *Hermetia illucens* Larval Survival Over 72-hours

The survival of *Hermetia illucens* was monitored over 24, 48, and 72 hours post-injection with 10 µL injections of Saline (0.85% NaCl), PBS (Phosphate-Buffered Saline) and an injection-only control (no volume) to assess the physiological impact of different suspension media on larval viability (Figure 2.2-2.3; Table 2.5-2.6)

Larvae injected with saline exhibited the lowest survival rates across all time points: 84.34% at 24 hours, 61.45% at 48 hours, and 51.67% at 72 hours. These results suggest unbuffered saline may induce physiological stress, leading to a steady decline in viability. The magnitude of these differences was biologically meaningful, with over 30% more larvae surviving in the PBS group than in the saline group by 72 hours. Statistical analysis (Table 2.6) confirmed a significant difference in survival between saline and PBS ($\chi^2 = 16.71$, $p = 0.000235$ at 24 h), which became even more pronounced at 48 h ($\chi^2 = 19.45$, $p = 0.000060$) and 72 h ($\chi^2 = 18.08$, $p = 0.000118$). This strongly suggests that the unbuffered nature of saline negatively impacted larval viability.

One of the most likely reasons for increased mortality in the saline-injected larvae, compared to PBS and injection-only controls, is the lack of pH buffering in saline solutions. 0.85% saline (NaCl in water) has no buffering capacity and can have a pH ranging between 5.5 and 7.0, depending on factors like CO₂, absorption from the air, and ion interactions. *Hermetia illucens*, like many insects, relies on a relatively stable physiological pH (usually close to neutral, ~7.2-7.5) for optimal enzymatic function and cellular processes. If the injected saline is too acidic (pH closer to 5.5-6.0), it could cause cellular stress, disrupt metabolic activity, and reduce haemolymph pH (acidosis), further contributing to increased mortality.

In contrast, larvae injected with PBS showed markedly improved survival: 96% at 24 hours, 72% at 48 hours, and 70.67% at 72 hours. PBS is pH-stabilised (typically pH 7.2-7.4) and mimics insect haemolymph conditions more effectively than saline, preventing sudden drops in pH and associated metabolic stress. Statistical analysis confirmed

that PBS-treated larvae had significantly higher survival rates than saline-injected larvae at all time points ($p < 0.001$). However, PBS survival remained slightly lower than the injection-only control ($\chi^2 = 6.41$, $p = 0.011$ at 72h), suggesting that while PBS reduces physiological stress compared to saline the injection procedure still introduces a minor However, PBS survival remained slightly lower than the injection-only control ($\chi^2 = 6.41$, $p = 0.011$ at 72h), suggesting that while PBS reduces physiological stress compared to saline the injection procedure still introduces a minor but measurable physiological response.

The Injection-only groups exhibited the highest survival across all time points, with 100% survival at 24 hours, 92% at 48 hours and 84% at 72 hours. This highlights that the mechanical stress of needle puncture is comparatively minor and that the chemical composition of the injected solution is the primary determinant of mortality risk. The significantly higher survival rates in the injection-only group compared to saline-injected larvae ($\chi^2 = 24.67$, $p = 0.000004$ at 72 h) confirm that saline is the primary driver of reduced survival, rather than the injection process alone.

While mortality was carefully recorded, it is acknowledged that a small proportion of larvae may have entered the prepupal stage earlier than expected, potentially complicating visual assessment of viability. Nonetheless, these findings strongly indicate that PBS is the most suitable injection medium, as it minimises physiological stress and improves larval viability, making it the preferred choice for immune-related studies in *H. illucens*.

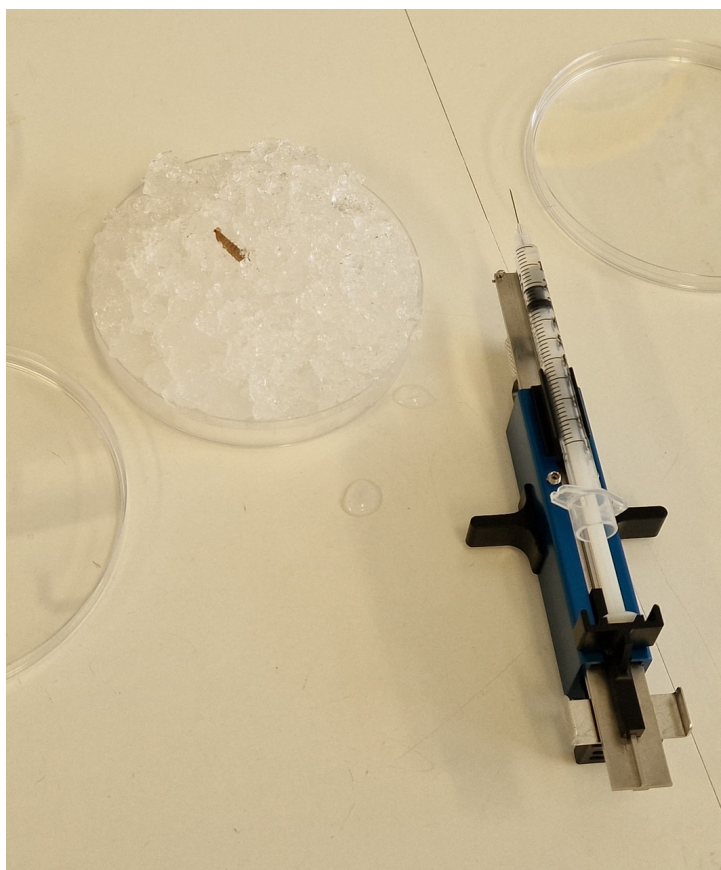


Figure 2.2 *Hermetia illucens* larvae anaesthetised on ice before injection. Pre-injection chilling is used to slow larval movement and reduce stress

Table 2.5 Survival rates of *Hermetia illucens* larvae following injection with saline, PBS, or injection-only control over 24, 48, and 72 hours

Injection Type	N=	24 hrs Survival (%)	24 hrs Alive	24 hrs Dead	48hrs Survival (%)	48hrs Alive	48hrs Dead	72hrs Survival (%)	72hrs Alive	72hrs Dead
Saline	75	84.34	63	12	61.45	46	29	51.67	39	36
PBS	75	96.0	72	3	72.0	54	21	70.67	53	22
Injection Only (No Volume)	75	100.0	75	0	92.0	69	6	84.0	63	12

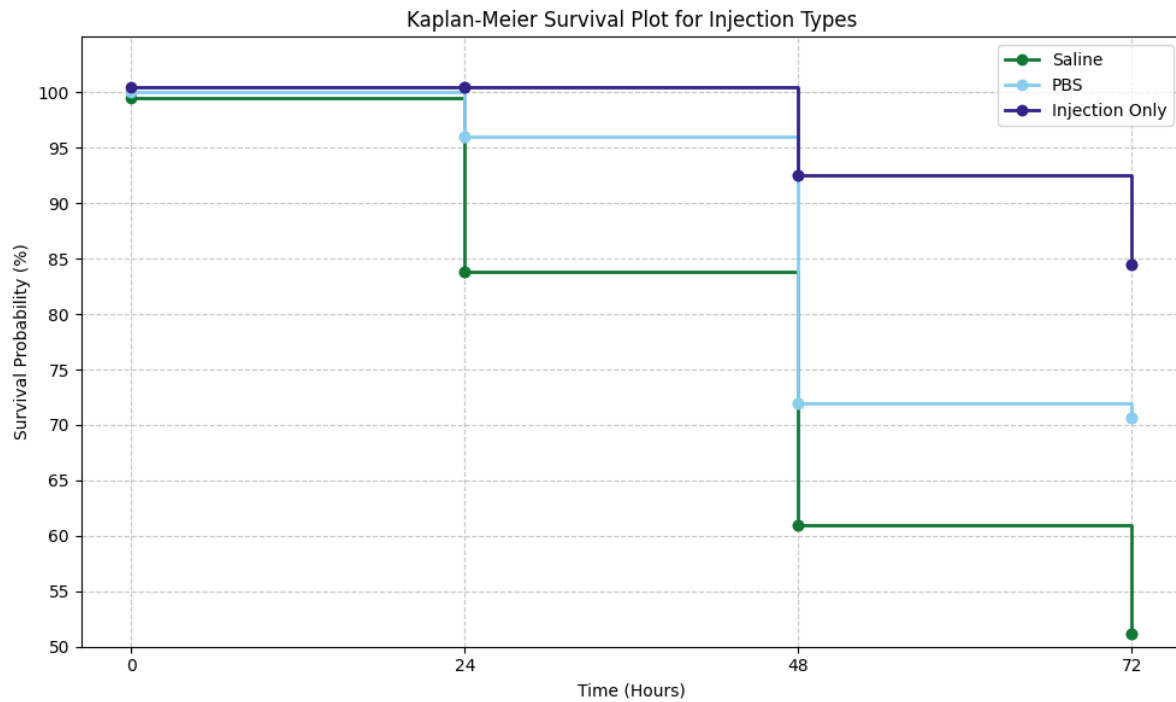


Figure 2.3 Survival rates of *Hermetia illucens* larvae following injection with saline, PBS, or injection-only (no volume) control over 24, 48, and 72 hours using Python's Matplotlib library

Table 2.6 Chi-square results showing comparisons between Saline, PBS, and Injection only over 24 h, 48h, and 72h

Comparison	24 h Chi-Square Statistic	24 h P-Value	48h Chi-Square Statistic	48h P-Value	72h Chi-Square Statistic	72h P-Value
Saline vs PBS	16.71	0.000235	19.45	6.00E-05	18.08	0.000118
Saline vs Injection Only	22.45	2.00E-05	28.12	1.00E-06	24.67	4.00E-06
PBS vs Injection Only	5.23	0.022	7.34	0.009	6.41	0.011

2.17. Determining the impact of larval weight on survival after Pathogen-associated molecular pattern (PAMP) exposure

The survival analysis of *Hermetia illucens* larvae injected with pathogen-associated molecular patterns (PAMPs), lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (Poly I:C) was conducted using Kaplan-Meier survival curves. Larvae were grouped by weight class ($100\text{ mg} \pm 20\text{ mg}$ and $200\text{ mg} \pm 20\text{ mg}$) at 12 days post-hatching and injected with four concentrations of each PAMP: $2\text{ }\mu\text{g}$, 200 ng , 20 ng , and 2 ng . Survival was monitored over 24 hours. The data demonstrate a clear weight-dependent effect, with larger larvae exhibiting greater resilience to immune challenge (Figure 2.4-2.5).

Across all concentrations in the 100 mg group, Poly I:C and LPS treatments resulted in markedly lower survival compared to Control and Injection Only (IO) groups (Appendix Table 1-3). At 24 hours, the most substantial reductions were observed in Poly I:C-treated larvae, where survival declined to 77.78% at both $2\text{ }\mu\text{g}$ and 200 ng , and 75.56% at 20 ng and 2 ng . This consistent decline across doses suggests that Poly I:C-induced mortality was not strictly dose-dependent. Instead of a linear dose-response relationship, mortality appeared to plateau after initial immune activation, indicating that even low concentrations were sufficient to trigger a systemic response with associated physiological costs.

LPS-treated larvae also showed reduced survival, although the response was more variable. At $2\text{ }\mu\text{g}$, survival was 83.22% at 24 hours, while survival rates at 200 ng (76.67%), 20 ng (81.11%), and 2 ng (77.78%) remained in a similar range. As with Poly I:C, there was no clear dose-response relationship, indicating that even low levels of LPS can elicit a measurable physiological cost.

PBS-injected larvae showed moderate reductions in survival across all concentrations, with 24-hour survival ranging from 89.89% ($2\text{ }\mu\text{g}$) to 78.89% (2 ng), suggesting a physiological response to the suspension medium or the injection procedure itself, especially in smaller larvae.

In contrast, 200 mg larvae exhibited significantly higher survival across all treatments and concentrations (Figure 2.5). Although Poly I:C still resulted in the lowest survival within this group, the effect was markedly attenuated. At 2 µg, survival remained at 90%, compared to 77.78% in 100 mg larvae. Similar trends were observed at 200 ng (92.22%), 20 ng (95.56%), and 2 ng (96.67%).

PBS-injected 200 mg larvae also exhibited improved survival compared to their 100 mg counterparts, maintaining 96.67% survival at 2 µg and 90% at 200 ng, with minimal mortality at lower concentrations.

LPS-treated larvae also showed statistically higher survival in the 200 mg group across all concentrations. Log-rank analysis revealed significant differences between weight classes for all doses tested ($p < 0.05$), highlighting a protective effect of larger body size against PAMP-induced stress or mortality.

Log-rank tests comparing survival curves between weight classes confirmed that Poly I:C-treated larvae had significantly lower survival in the 100 mg group than in the 200 mg group at 2 µg ($p < 0.01$). Similar significant differences were observed at 200 ng ($p = 0.015$), 20 ng ($p = 0.008$), and 2 ng ($p = 0.003$). PBS-injected larvae also showed statistically lower survival in the 100 mg group at 2 ng ($p = 0.005$) and 20 ng ($p = 0.047$), suggesting that injection-related stress may contribute disproportionately to mortality in smaller larvae.

Together, these results suggest that *Hermetia illucens* larvae exhibit a weight-dependent resilience to immune stimulation, with smaller larvae more susceptible to both immune-related and injection-associated mortality. The findings support the use of weight-matched individuals in immunological challenge assays and highlight the importance of considering body mass when interpreting dose-responsiveness in larval insect models.

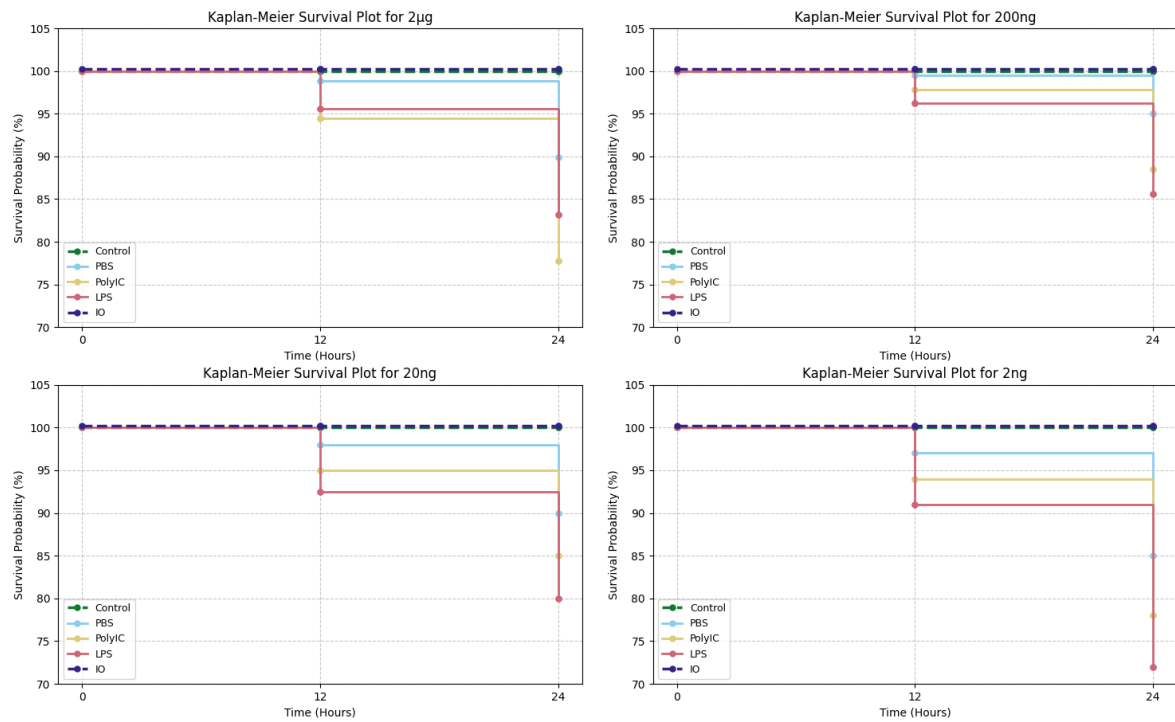


Figure 2.4 Kaplan-Meier survival curves of 100 mg *Hermetia illucens* larvae over 24 hours following injection with different concentrations of PAMPs (2µg, 200ng, 20ng, 2ng). Treatments included Poly I:C, LPS, PBS, and Injection Only (IO) controls.

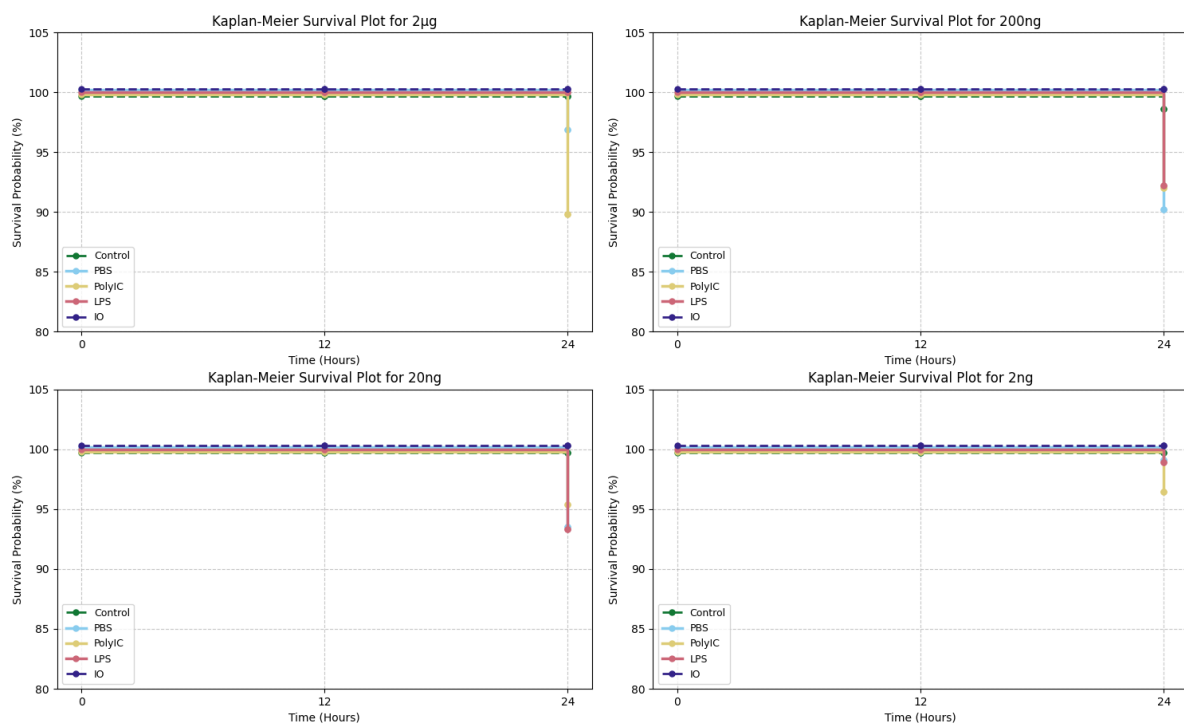


Figure 2.5 Kaplan-Meier survival curves of 200 mg *Hermetia illucens* larvae over 24 hours following injection with different concentrations of PAMPs (2µg, 200ng, 20ng, 2ng). Treatments included Poly I:C, LPS, PBS, and Injection Only (IO) controls.

2.18. Extracting RNA from small (80-125mg) and big (200-220mg) whole-body larvae following TRIzol manufacturing instructions

totalRNA extracted from whole-body *Hermetia illucens* larvae was performed using the TRIzol method (Figure 2.6A-C), following the manufacturer's instructions. The extracted RNA was assessed for integrity using agarose gel electrophoresis (1x TAE buffer), as shown in Figure 2.7. Under ideal conditions, insect RNA should primarily display the 18S rRNA band, as the 28S rRNA undergoes degradation and comigration under denaturing conditions and is typically not visible. However, the results from this initial extraction revealed significant smearing, additional unexpected bands, and inconsistent RNA integrity across samples, indicating possible degradation, contamination, or inefficient RNA recovery. Furthermore, this can be reflected by A260/A280 and A260/A230 values, suggesting severe phenol or guanidine salt contamination (Appendix; Table 4).

These issues posed several concerns for downstream qPCR analysis, where RNA quality directly impacts gene expression results. Contaminating genomic DNA, degraded RNA, and residual proteins or phenol can all interfere with cDNA synthesis efficiency and qPCR amplification, leading to erroneous gene expression data, inflated Ct values, or non-specific amplification (Padhi, Singh, Rosales, Pelletier, & Cakmak, 2018) (Palladino, et al., 2015). The presence of additional bands or smearing in the gel suggests that RNA fragmentation or incomplete phase separation during extraction may have led to the co-purification of unwanted cellular components, affecting overall RNA purity and stability.

During the phase separation step, many samples exhibited carryover of phenol, visible as a pink hue in the aqueous layer (Figure 2.6A). Effective RNA extraction relies on precise phase partitioning to isolate high-purity RNA while excluding proteins, lipids, and organic solvents. Following BCP/Chloroform addition and centrifugation, a clean extraction should yield a clear, colourless aqueous phase containing RNA, while proteins and lipophilic impurities remain in the interphase and organic layer. In this

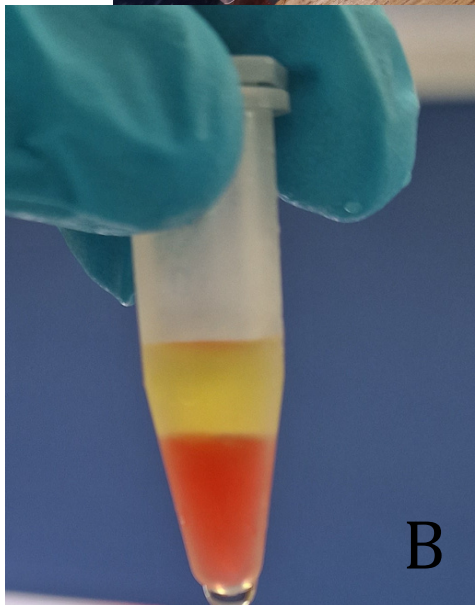
study, incomplete phase separation likely contributed to co-purification of contaminants, as reflected in low 260/230 ratios and gel smearing. These issues can interfere with downstream applications such as reverse transcription and qPCR, underlying the need for refined separation steps and consistent sample input mass.

In addition to this, larger larvae (>200 mg) were noted to have a yellow hue during the aqueous phase (Figure 2.6B), which could be attributed to fat or chitin carryover, and many samples had an RNA pellet so large (Figure 2.6C) that it was unable to resuspend in dH₂O. Suboptimal phase separation observed in several samples is likely responsible for the variability in RNA integrity seen in Figure 2.7, emphasising the need for optimised extraction conditions. Notably, the last eight samples in Figure 2.6A display a clear aqueous phase, corresponding with higher RNA integrity as observed on the gel and A260/A280 and A260/A230 ratios.

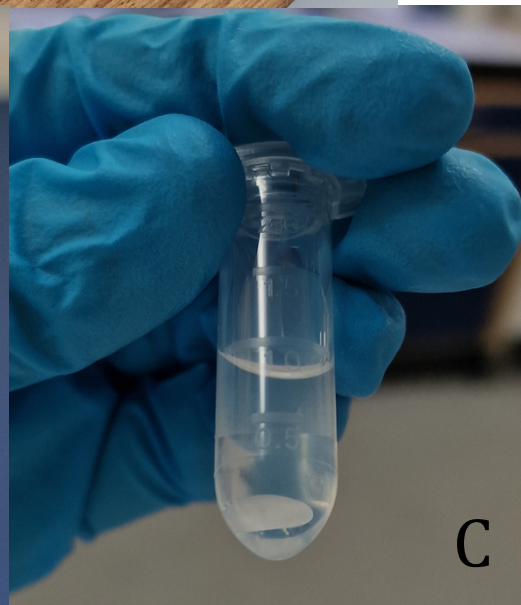
This highlights the critical importance of achieving proper phase separation to ensure high-quality RNA for gene expression studies, and therefore, the need for an optimised protocol that ensures consistently high-quality RNA across all experimental conditions.



A



B



C

Figure 2.6 (A) Partial Separation of aqueous phase. (B) Discoloured aqueous phase present in larger larvae (>200mg), potentially due to fat or chitin carryover. (C) large RNA pellet unable to resuspend in dH_2O .

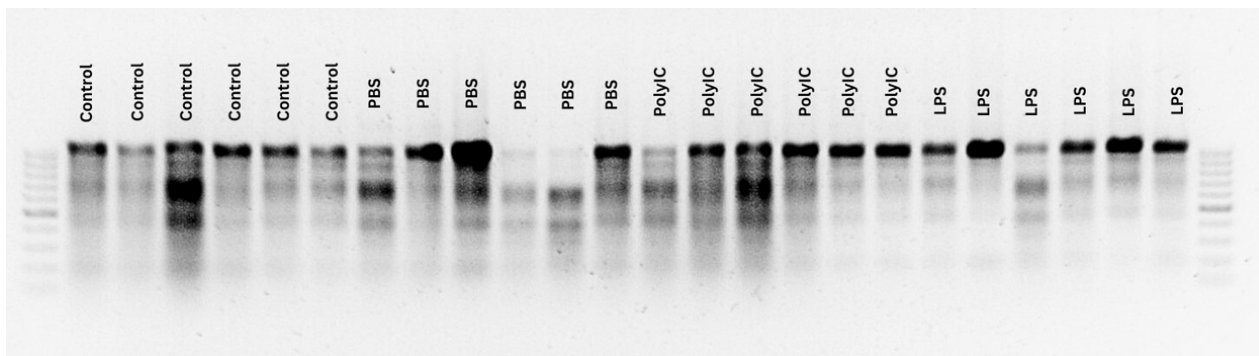


Figure 2.7 RNA integrity assessed via 1% agarose gel electrophoresis (1× TAE buffer). Each lane contains RNA extracted from individual larvae across treatment groups. While many samples show defined 18S bands, smearing and additional bands are visible in others, indicating partial degradation, co-purified contaminants, or inconsistent RNA recovery.

2.19. Optimising of RNA extraction: Evaluating TRI Volumes, Homogenization Techniques, and Phase Separation Reagents in small and large *Hermetia illucens* larvae

Following the identification of poor RNA purity. Smearing on gels and persistent issues with phase separation in initial extractions, further protocol optimisations were undertaken to improve overall RNA yield, integrity, and resuspension efficiency. This round of optimisation focused on four main variables: Volume of TRI reagent (500ml vs 1000ml), homogenisation method (with or without ceramic beads during mechanical homogenisation using a bead beater), phase separation reagent (chloroform vs 1-bromo-3-chloropropane [BCP]), and resuspension volume (increased to 150ml nucleus-free water). Both large (~200 mg; "BIG") and smaller (60–120 mg; "SMALL") larvae were tested to evaluate whether input mass influenced extraction success under these modified conditions.

Gel electrophoresis results (Figure 2.8) showed notable improvements in RNA integrity across most experimental conditions. The clearest 18S rRNA bands were observed in samples extracted from smaller larvae using 500 μ L of TRI and bead-assisted homogenisation. These lanes displayed high molecular weight RNA with minimal smearing, suggesting reduced degradation and more consistent extractions.

While both chloroform and BCP successfully facilitated phase separation, no clear advantage of BCP over chloroform was observed based on gel visualisation alone. However, some samples processed with BCP showed marginally improved 260/280 ratios, suggesting slightly reduced protein contamination, although A260/230 ratios remained suboptimal in many cases. These low 260/230 values indicate that phenol or guanidine salt contamination was still present, likely due to either inefficient aqueous phase separation or incomplete RNA pellet resuspension.

Notably, larger larvae continued to pose challenges during the resuspension phase. In several "BIG" samples, the RNA pellet was visibly large and dense (as previously shown in Figure 2.6C), and despite increasing the resuspension volume to 150 μ L, complete

solubilisation was not always achieved. This may have led to underestimation of total yield via Nanodrop and could explain the lingering contamination signals.

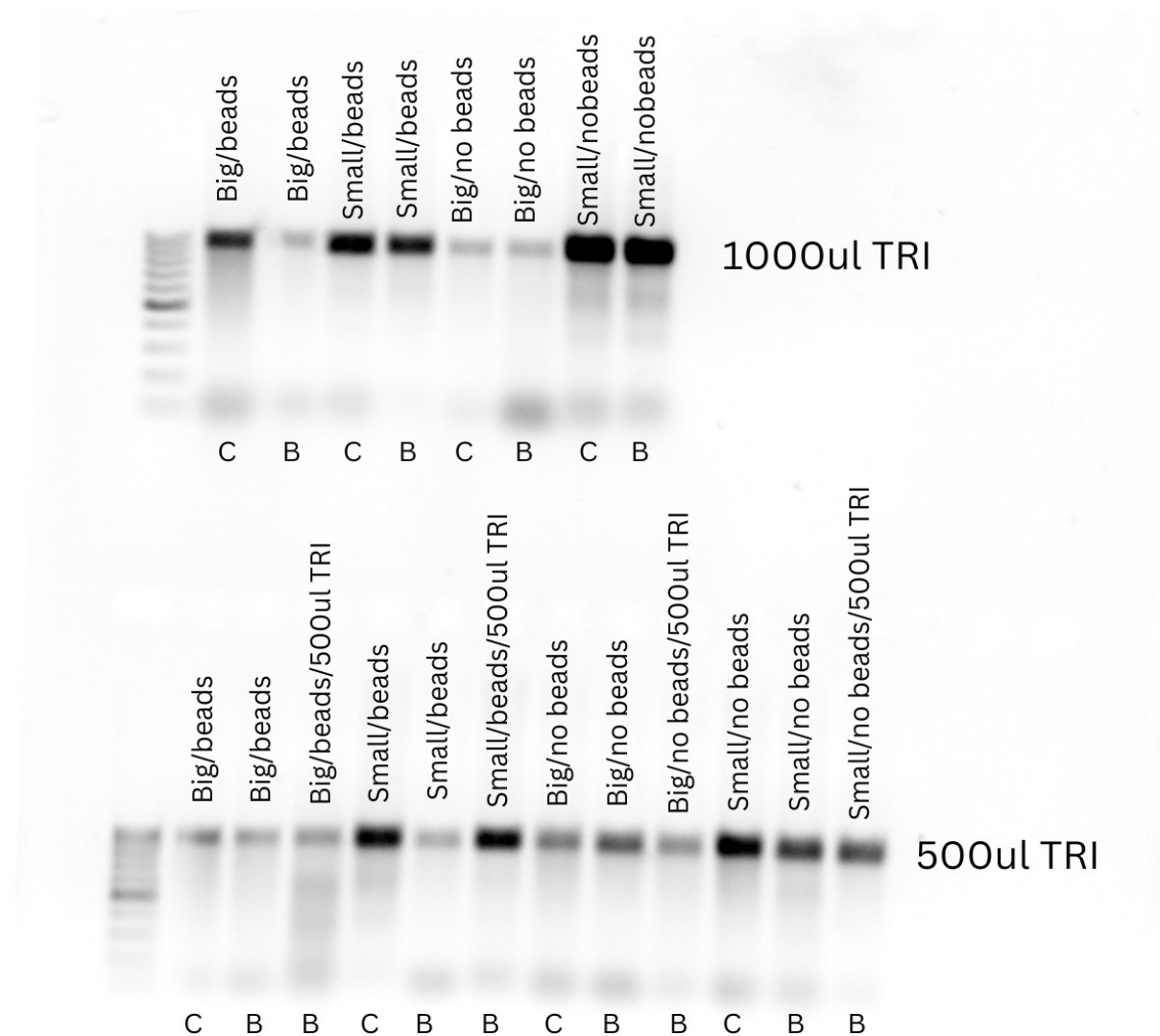


Figure 2.8 Comparison of RNA integrity following different homogenisation methods and Trizol volume, and phase separation techniques in *Hermetia illucens* larvae

2.20. Further optimisation: Increasing ethanol washes and resuspension techniques

To further refine RNA extraction quality, a focused comparison was performed between two and three ethanol wash steps during the RNA precipitation phase (Figure 2.10; Tables 2.8-2.9). Ethanol washing is a critical purification step in the TRIzol protocol, used to remove residual salts, phenol, and organic contaminants that interfere with downstream applications. However, excessive washing can also risk RNA loss, particularly in small-scale extractions. Therefore, identifying the optimal number of washes was essential to maximise RNA purity without compromising yield. RNA was extracted from *Hermetia illucens* whole-body larvae and subjected to either two or three 75% ethanol washes, followed by resuspension in 150 µL of nuclease-free water. The resulting RNA was assessed for concentration, purity (A260/A280 and A260/A230), and visual integrity on a 1% agarose gel.

Figure 2.9 presents a side-by-side comparison of the RNA extracted under the two conditions. Samples that underwent three ethanol washes (right panel) exhibited markedly cleaner banding patterns, with well-defined 18S rRNA bands and noticeably less background smearing or streaking, indicative of reduced contamination. Quantitative spectrophotometric analysis (Table 2.7) revealed a consistent improvement in RNA purity with the three-wash protocol: A260/A230 ratios, which indicate contamination with phenol, guanidine salts, or carbohydrates, were significantly higher in the three-wash group (2.03 ± 0.05) compared to two washes (1.88 ± 0.17).

A260/A280 ratios, which reflect protein contamination, were also improved with three washes (2.17 ± 0.10 vs. 1.86 ± 0.07), indicating better removal of protein or organic carryover.

Importantly, these improvements in purity were not accompanied by a reduction in RNA yield. In fact, the three-wash protocol slightly increased the average RNA concentration to 426.49 ± 86.53 ng/µL, compared to 339.52 ± 110.15 ng/µL in the two-wash group

(Table 2.8). This suggests that an additional ethanol wash does not compromise yield and may even facilitate more effective removal of contaminants that interfere with accurate quantification.

Taken together, these findings indicate that three ethanol washes offer superior RNA purification in BSFL samples, producing RNA of higher purity and visual integrity without negatively affecting yield. This optimisation is particularly important given the high lipid and chitin content of *H. illucens* larvae, which increases the risk of carryover contaminants during organic extraction.

The adoption of a three-wash protocol is therefore recommended as the standard step in future extractions from *H. illucens*, particularly when high-quality RNA is required for sensitive downstream applications such as quantitative PCR or RNA sequencing.

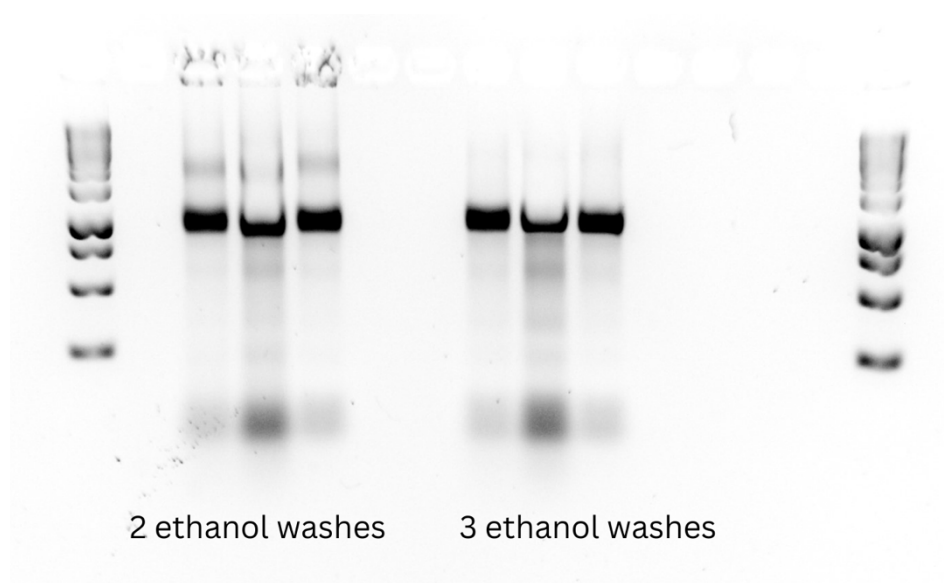


Figure 2.9 Optimizing purification steps for RNA extraction in *Hermetia illucens*

Table 2.7 Quantitative spectrophotometric analysis of RNA purity values based on 2 or 3 75% ethanol washes

	ng/ul	260/230	260/280
2_E_1	430.11	1.67	1.8
2_E_2	211.45	1.97	1.85
2_E_3	376.99	1.99	1.93
3_E_1	513.26	2	2.3
3_E_2	344.23	1.98	2.1
3_E_3	421.99	2.1	2.1

Table 2.8 Effect of additional ethanol wash on RNA yield and purity in *Hermetia illucens* larvae.

Washes	RNA Yield (ng/μL) (Mean ± SD)	A260/A230 (Mean ± SD)	A260/A280 (Mean ± SD)
2 Ethanol Washes	339.52 ± 110.15	1.88 ± 0.17	1.86 ± 0.07
3 Ethanol Washes	426.49 ± 86.53	2.03 ± 0.05	2.17 ± 0.10

2.21. RNA Yield and Purity Across Different Extraction Methods

To identify the most effective RNA extraction approach for *Hermetia illucens* larvae, three protocols were compared: the RNeasy Mini Kit (Qiagen), the standard TRIzol protocol, and an amended BSFL TRIzol protocol developed through further optimisation. Key metrics were re-evaluated to determine if either kit or amended TRIzol protocol would be suitable for downstream analysis.

As shown in Figure 2.10, RNA concentration differed markedly between methods. The RNeasy Mini Kit produced the lowest yield at 120 ng/μL, followed by standard TRIzol at 201 ng/μL. The amended BSFL TRIzol protocol yielded the highest RNA concentration at 311 ng/μL, a 2.6-fold increase over the kit-based method. This improved yield is likely attributed to several modified steps, including longer phenol incubation, additional extraction per larva (3 longitudinal extractions per larva), optimised phase separation, increased ethanol wash steps, and a higher resuspension volume/heating, all of which

enhanced lysis efficiency and pellet solubilisation. In addition, due to the increased lipid content, column-based extraction kits may be more susceptible to blockage, preventing higher RNA yields.

RNA purity, as determined by the A260/280 ratio, is a critical metric for evaluating protein or phenol contamination. As shown in Figure 2.11, the amended BSFL TRIzol protocol outperformed both other methods with a 260/280 ratio of 2.05, slightly higher than the RNeasy Mini Kit (2.01) and substantially better than the standard TRIzol protocol (1.80). The lower value in the standard TRIzol method suggests incomplete protein removal or residual phenol contamination due to insufficient phase separation or ethanol purification.

As shown in Figure 2.12, both the RNeasy Mini Kit and standard TRIzol protocols achieved moderate purity (1.90), while the amended BSFL TRIzol protocol demonstrated a notable improvement, reaching a 260/230 ratio of 2.10. This suggests more effective removal of co-extracted contaminants.

These results collectively demonstrate that the amended BSFL TRIzol protocol significantly improves RNA yield and purity compared to both the commercial kit and the standard manufacturer-recommended TRIzol protocol. While the RNeasy Mini Kit is known for producing clean RNA, it is typically less efficient in recovering total RNA from whole insect tissues, particularly those with high lipid and chitin content such as *H. illucens*.

In contrast, the amended BSFL TRIzol protocol balances both yield and quality, making it highly suitable for downstream applications requiring substantial RNA input and high purity, including reverse transcription, qPCR, and transcriptomic analyses. These findings support the continued use and further refinement of the TRIzol-based method in future *H. illucens* RNA studies, particularly in contexts where cost-effectiveness, scalability, and total RNA yield are critical.

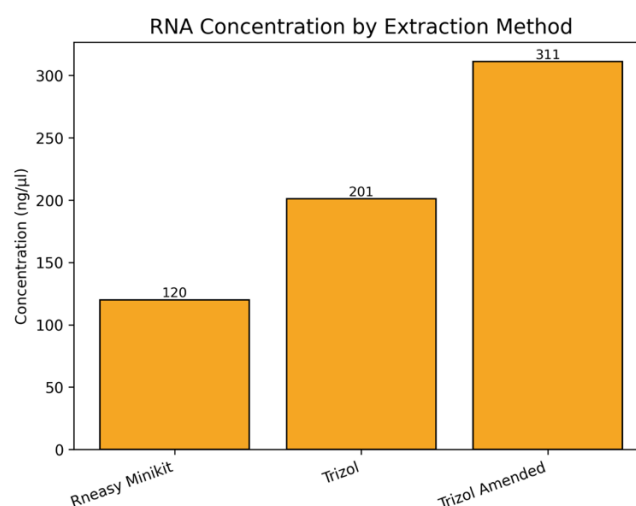


Figure 2.10 RNA was extracted using TRIZol, Amended TRIZol, and the RNeasy Mini Kit. Amended TRIZol resulted in the highest RNA yield, indicating improved extraction efficiency. RNeasy Mini Kit yielded significantly lower RNA concentrations, likely due to column clogging from high-fat and chitin content in *Hermetia illucens* larvae. Error bars represent standard deviation (SD)

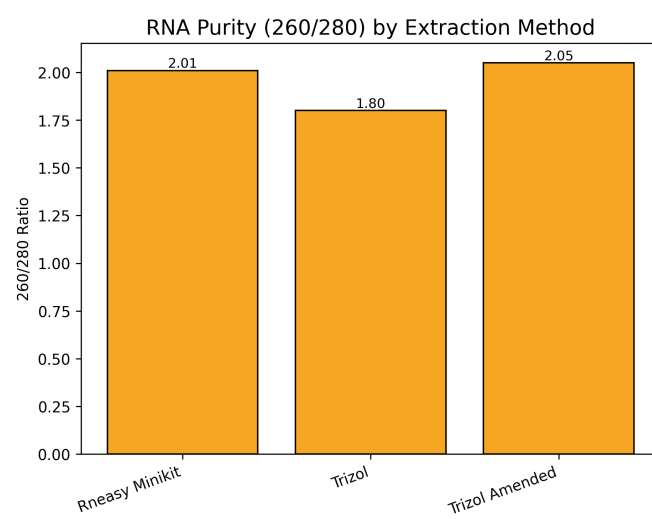


Figure 2.11 The A260/A280 ratio assesses protein contamination, with optimal values around ~2.0. Amended TRIZol yielded the most consistent purity, while standard TRIZol showed greater variability, indicating occasional protein or phenol contamination. RNeasy Mini Kit had inconsistent values, likely due to inefficient lysis of high-lipid tissue.

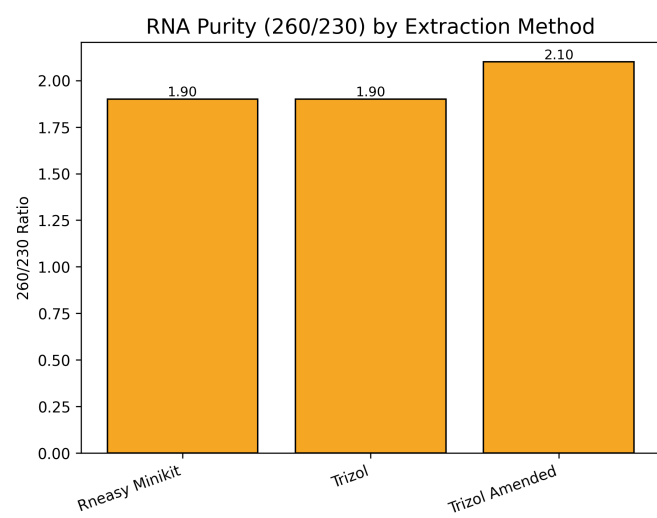


Figure 2.12 The A260/A230 ratio evaluates phenol and salt contamination, where values above 2.0 are preferred. Amended TRIzol showed the highest and most consistent values, indicating effective phenol removal. Standard TRIzol exhibited lower values (~1.84–1.99), suggesting phenol carryover, while RNeasy Mini Kit showed the lowest ratios (~1.44–1.86), indicating residual salt or organic contaminants

2.22. Comigration of 28s and 18s in denatured RNA in *Hermetia illucens*

To confirm RNA integrity and examine the characteristic behaviour of insect ribosomal RNA under different electrophoretic conditions, total RNA from *Hermetia illucens* larvae was analysed via agarose gel electrophoresis under both non-denaturing and denaturing conditions (Figure 2.13). Non-denatured samples showed two prominent bands, corresponding to the 28S and 18S ribosomal RNA subunits.

In contrast, when the same samples were run under denaturing conditions, only a single prominent rRNA band was detected, corresponding to 18S rRNA. The apparent disappearance or merging of the 28S rRNA band is a well-documented phenomenon in insect species, attributed to the intrinsic fragility of the 28S molecule and the presence of hidden breaks. These breaks cause the 28S rRNA to denature into fragments that migrate at approximately the same rate as 18S rRNA, resulting in their comigration on denaturing gels (Macharia, Ombura, & Aroko, 2015) (Jowett, 1986) (Fujiwara & Ishikawa, 1986). This pattern is frequently reported in insect studies and should not be misinterpreted as RNA degradation. Instead, it reflects a species-specific structural feature of insect rRNA and is important to consider during downstream RNA quality

assessments. This observation confirms the expected rRNA behaviour in *H. illucens* and supports the validity of using 18S rRNA as a marker for RNA integrity in this species, particularly under denaturing conditions where 28S rRNA may be masked or fragmented.

Due to the dense nature of RNA pellets obtained from *Hermetia illucens*, full resuspension was challenging even in 150 µL of nuclease-free water. To enhance RNA recovery, a brief incubation at 65 °C for 5 minutes post-elution was introduced, promoting effective pellet solubilisation while preserving RNA quality and structural integrity for downstream analysis.

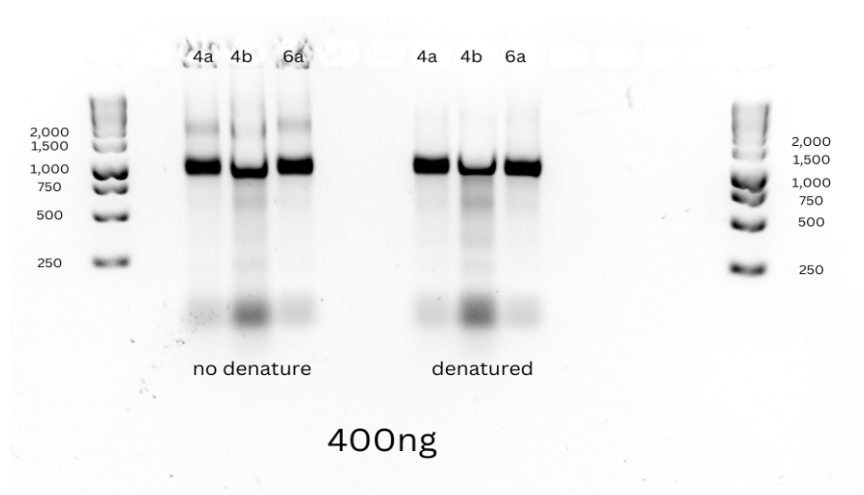


Figure 2.13 Observation of the “Hidden Break” in the 28S subunit in Insects. *Hermetia illucens* larvae RNA assessed under non-denaturing and denaturing conditions, showing co-migration of the 28S and 18S subunits.

2.23. RNA extractions ready for downstream qPCR analysis

Before pooling, all RNA extractions underwent individual quantification and quality assessment to determine their suitability for downstream applications such as library preparation and transcriptome sequencing. This included evaluation of RNA yield (ng/mL) and purity via spectrophotometric absorbance ratios at 260/280 nm and 260/230 nm (Figures 2.14-16). Only samples that met established purity thresholds for both 260/280 and 260/230 ratios were carried forward for downstream processing.

RNA concentration across all extractions followed a positively skewed (right-skewed) distribution, with the majority of samples falling between 100-400ng/mL and a smaller number of high-yield outliers extending above 600ng/mL. This skew is reflected by a mean (306.36 ng/mL) that is higher than the median (287.75ng/mL).

The 260/280 absorbance ratio is a widely used indicator of protein contamination, with pure RNA typically exhibiting values between 2.0-2.2. In this dataset, 260/280 ratios followed a roughly normal distribution centred around 2.05 (mean) and 2.06 (median), with most values falling within the acceptable range (Figure 2.15). A small number of samples showed values below 1.9 or above 2.2, potentially reflecting residual phenol protein or reagent interference. Those falling outside this range were flagged for potential exclusion, and if needed, were re-extracted.

The 260/230 ratio serves as an indicator of contamination from carbohydrates, salts, or organic compounds such as guanidinium or phenol. Ideal values range between 2.0-2.4 for high-quality RNA. As shown in Figure 2.16, 260/230 ratios were broadly distributed with a mean of 2.11 and a median of 2.13.

To ensure data integrity and minimise downstream technical noise, only samples that exhibited 260/280 values between 1.9 and 2.2 and 260/230 values between 2.0 and 2.4 were included for subsequent pooling and library preparation. Samples falling outside these thresholds were excluded unless their deviation was minimal and corroborated by strong RNA yield and consistent gel electrophoresis results (data not shown). This rigorous quality control ensured that only high-purity RNA contributed to the pooled transcriptomic samples, improving the reliability of expression data and minimising artefacts introduced by contamination or degradation.

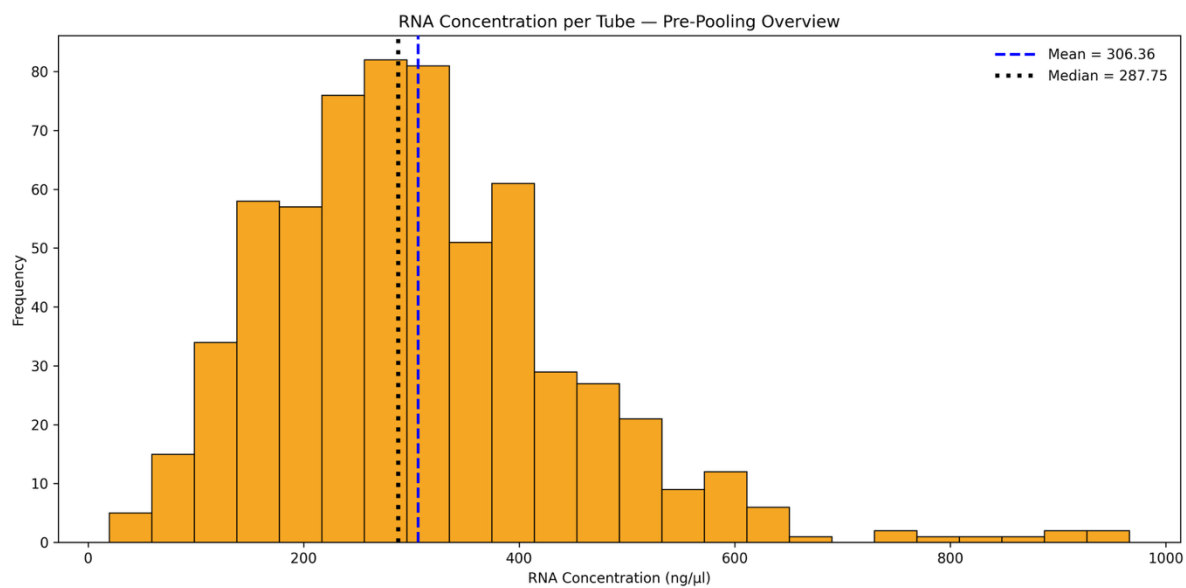


Figure 2.14 RNA concentration per extraction prior to pooling

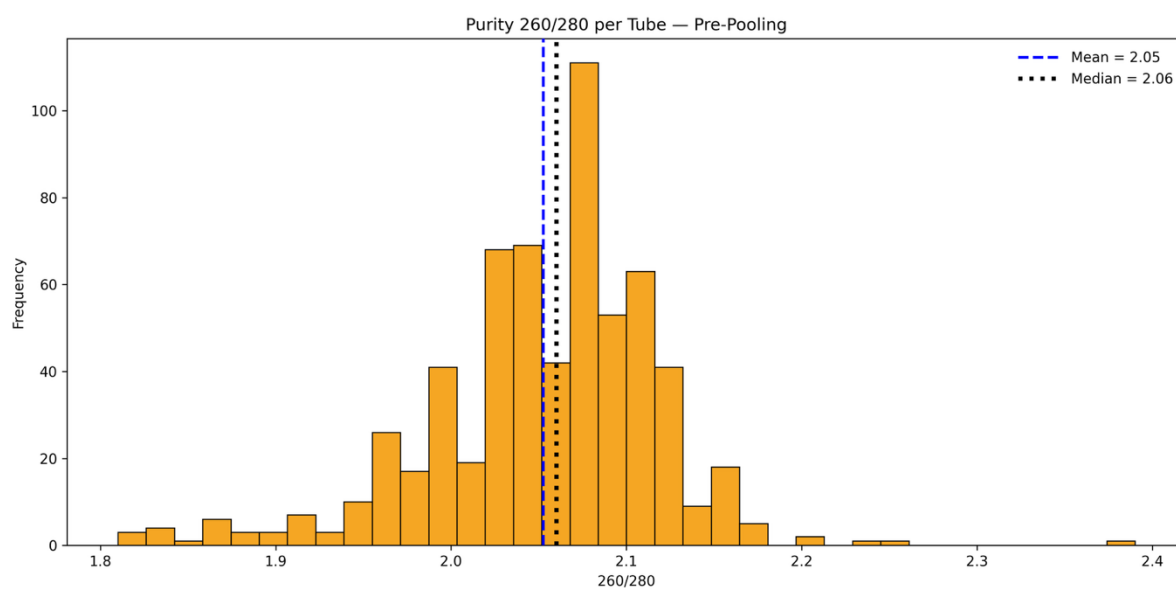


Figure 2.15 RNA purity (260/280 ratio) per extraction prior to pooling

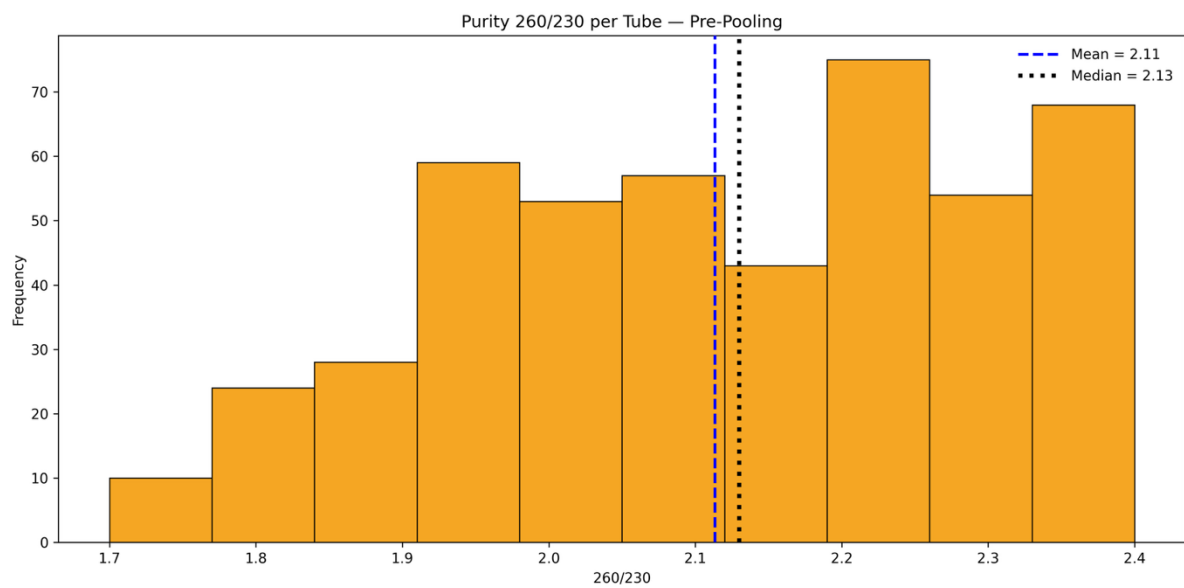


Figure 2.16 RNA purity (260/230 ratio) per extraction prior to pooling

2.24. Whole body Extractions (pooled RNA)

Following QC on individual dissections, three portions from the same larva were recombined to produce a single per-larva RNA sample (elution volume = 100 μ L). This approach reduces intra-larval variability and ensures that each final tube represents a complete larval transcriptome while retaining only dissections that met purity thresholds (260/280 between 1.9-2.2 and 260/230 between 2.0-2.4).

Per-larva concentrations were tightly centred around ~900ng/ μ L (mean = 907.63 ng/ μ L; median = 914.05 ng/ μ L), yielding ~90.8 μ g total RNA per larva (907.63 ng/ μ L x 100 μ L) (Figure 2.17). The small mean-median difference indicates minimal influence of outliers and consistent recovery across larvae, providing ample input for library construction.

260/280 ratios clustered narrowly around the expected RNA value (mean = 2.05, median = 2.06), with most samples between ~1.95-2.12, indicating negligible protein/phenol carryover after re-pooling of dissections (Figure 2.18).

260/230 Ratios were slightly broader (mean = 2.06, median = 2.11; ~1.4-2.4 overall), with the majority within the 2.0-2.4 target range (Figure 2.19). A minority of lower values likely reflects minor salt/buffer residues in one of the contributing dissections, but these did not materially affect the cohort averages.

Together, these data confirm that re-pooling three dissections from the same larva produced high-yield, high-purity per-larva RNA suitable for downstream transcriptomics. Only samples meeting the dual-purity criteria (260/280: 1.9–2.2; 260/230: 2.0–2.4) proceeded to library preparation.

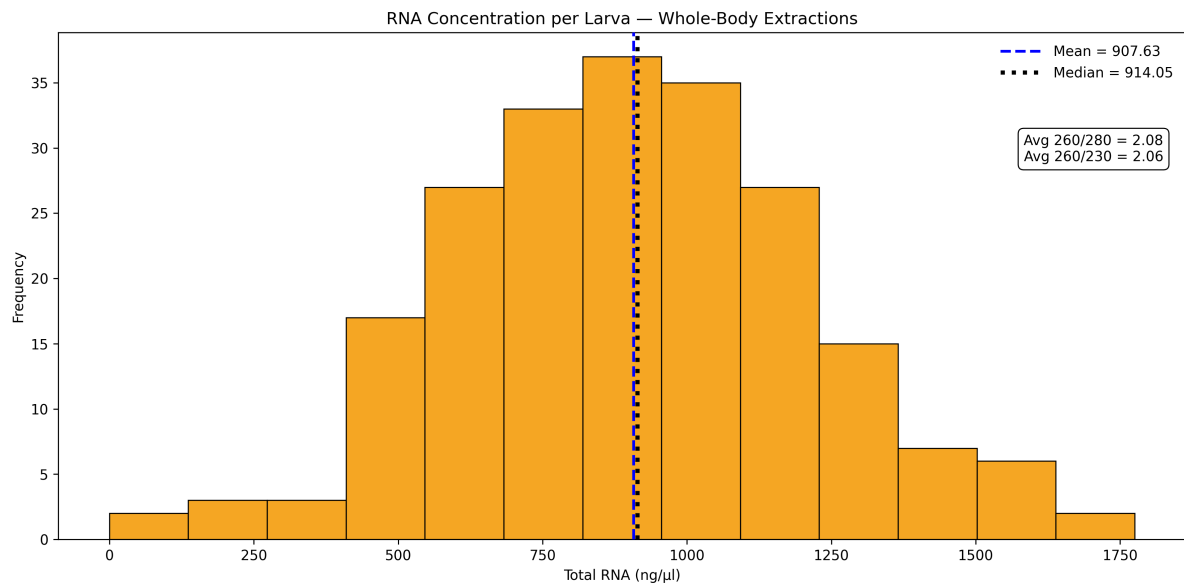


Figure 2.17 Total RNA concetration per larva following whole-body pooling of extractions

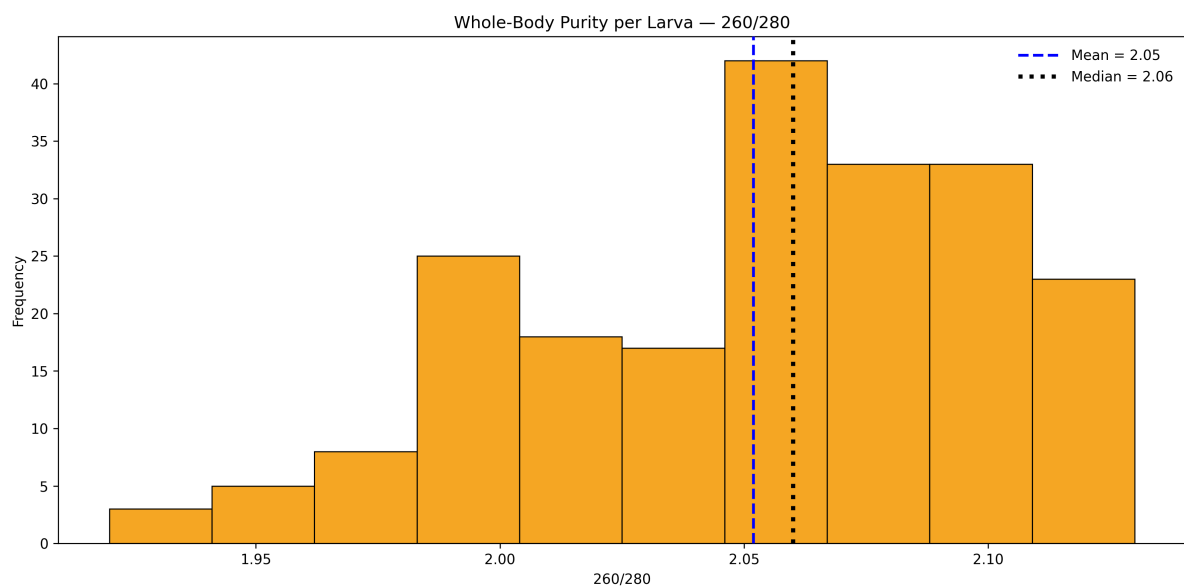


Figure 2.18 Purity assessment per larva (260/280 ratio) following pooling

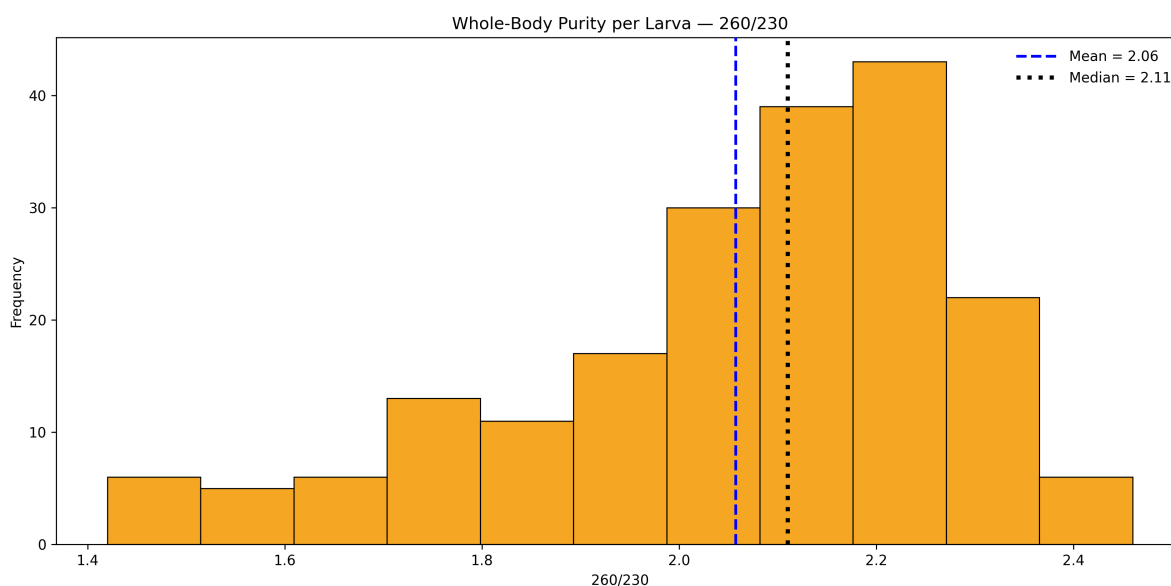


Figure 2.19 Purity assessment of larva (260/230 ratio) following RNA pooling

2.25. Assessment of Ribosomal RNA Profiles and Degradation Patterns in *Hermetia illucens*

In addition to Nanodrop quantification and gel electrophoresis to determine RNA integrity, RNA samples were also validated using the Agilent 2100 Bioanalyzer via RNA Nano chips (Cat. No. 5067-1511). While gel electrophoresis provides a quick and accessible visual check of RNA banding patterns, it has limitations in detecting partial degradation, particularly in insect species such as *Hermetia illucens*, where ribosomal RNA profiles can differ significantly from classical eukaryotic models due to structural features like the hidden break in the 28S rRNA.

The Bioanalyzer platform uses microfluidics-based separation and fluorescence detection to generate electropherograms and virtual gel images. Unlike agarose gels, which show size separation based on migration through a matrix, the Bioanalyzer measures electrophoretic migration time (in seconds) and fluorescence intensity, offering greater resolution and quantification. It also calculates an RNA Integrity Number (RIN), which scores RNA quality on a scale from 1 (fully degraded) to 10 (intact), based on peak shape, area, and the ratio of 28S to 18S rRNA signals.

This tool is especially useful in insect RNA quality assessment, where the 28S rRNA is prone to natural cleavage (the 'hidden break'), often resulting in fragmented 28S subunits that co-migrate with or appear near the 18S rRNA peak. As such, standard agarose gels may misleadingly suggest intact RNA even in partially degraded samples. The Bioanalyzer allows for detection of subtle degradation, smearing, or peak collapse that may not be evident on a gel, making it a critical QC step before qPCR, library prep and transcriptomic applications.

The Agilent Bioanalyzer displays RNA migration in terms of electrophoretic time (seconds), with intact 18S rRNA typically appearing at ~42–43 seconds and 28S rRNA at ~47–48 seconds, based on standard eukaryotic profiles (Mueller, Lightfoot, & Schroeder, 2004). In high-quality RNA samples, both peaks are clearly resolved, and the relative area between them contributes to the overall RIN score. However, in insect species such as *Hermetia illucens*, the hidden break in 28S rRNA fragmentation is exacerbated under microfluidic analysis due to heat, ionic detergents, and chaotropic agents that disrupt the non-covalent interactions between 28S subunits, causing them to migrate separately or collapse (Winnebeck, Millar, & Warman, 2010). This can lead to co-migration with the 18S fragment, formation of shoulders rather than distinct peaks, or a complete collapse of the 28S signal. As a result, 18S rRNA becomes the dominant observable feature in high-quality insect RNA, while the 28S signal may appear faint or absent even in intact samples.

Given these structural differences in insect rRNA, interpretation of RNA Integrity Numbers (RINs) requires careful contextualisation. In mammalian systems, a RIN value of ≥ 8 is typically used as the benchmark for high-quality RNA suitable for sensitive downstream applications such as RNA-seq or qPCR. However, in insect species such as *Hermetia illucens*, the inherent fragmentation of 28S rRNA due to the hidden break often leads to artificially lowered RIN scores, even when RNA is intact and functionally usable.

For this reason, RIN values as low as 7.0 are generally considered acceptable for insect total RNA, especially when other quality indicators, such as a sharp 18S rRNA peak,

minimal baseline noise, and lack of smearing on the virtual gel, are present. Samples with RIN values below this threshold, or those displaying broad peaks, high background, or the absence of a defined 18S peak, were excluded and re-extracted.

The Bioanalyzer image (Figure 2.20) confirmed that most RNA samples had visible rRNA bands, with stronger signals on 18S and a fainter upper band consistent with fragmented 28S. High-quality samples, such as LL15, LL20, LL34, LL39, and LL45, showed distinct banding with minimal smearing, indicative of good RNA integrity. In contrast, samples such as LL23 and LL47 showed diffuse or missing bands and extensive smearing across the lane, despite appearing relatively normal on initial agarose gels, highlighting the importance of a secondary QC step.

High-quality RNA profiles were characterised by clear, narrow 18S peaks with minimal baseline noise and a low presence of degraded RNA fragments (Figure 2.21). For example, samples LL15 (RIN 8.40), LL20 (RIN 8.60), LL34 (RIN 8.60), LL39 (RIN 9.00), LL42 (RIN 8.20), and LL45 (RIN 8.90) all displayed strong 18S signals. These samples showed clean baseline profiles with no evidence of significant degradation and were deemed highly suitable for downstream gene expression analysis.

By contrast, severely degraded or unusable samples displayed diffuse or collapsed peaks. LL23 (RIN 2.60) lacked any distinguishable 18S or 28S structure, instead exhibiting a broad, low-intensity smear throughout the run time, indicative of RNA degradation. LL47 (RIN: N/A) showed a small, featureless peak centred around 50–55 seconds, with no distinct ribosomal features. This suggests either advanced degradation or contamination, potentially from genomic DNA, phenol, or unprecipitated polysaccharides. These samples were excluded from downstream workflows and re-extracted under optimised conditions.

To assess the consistency and quality of RNA recovered using the optimised whole-body extraction protocol, RNA Integrity Numbers (RINs) were analysed across all processed samples. A total of 499 RNA samples were evaluated using the Agilent 2100

Bioanalyzer, and RIN values were plotted as a histogram with kernel density estimation to visualise their distribution (Figure 2.22).

The results demonstrate a clear right-skewed distribution, with most samples falling in the high-quality range. The mean RIN was 7.96, and the median RIN was 8.30, indicating that more than half of the samples exceeded the commonly accepted threshold for high-quality RNA ($\text{RIN} \geq 7.0$, shown as a red dashed line).

Notably, the histogram shows that most samples clustered between RIN 7.5 and 9.5, reflecting the reproducibility and effectiveness of the optimised protocol. A small number of lower-quality samples (<7.0) were also detected; however, these were identified and excluded from downstream workflows during the RNA quality control stage.

Together, these analyses underscore the necessity of species-specific RNA quality assessment protocols in insects such as *Hermetia illucens*, where conventional eukaryotic benchmarks may lead to the exclusion of functionally intact RNA.

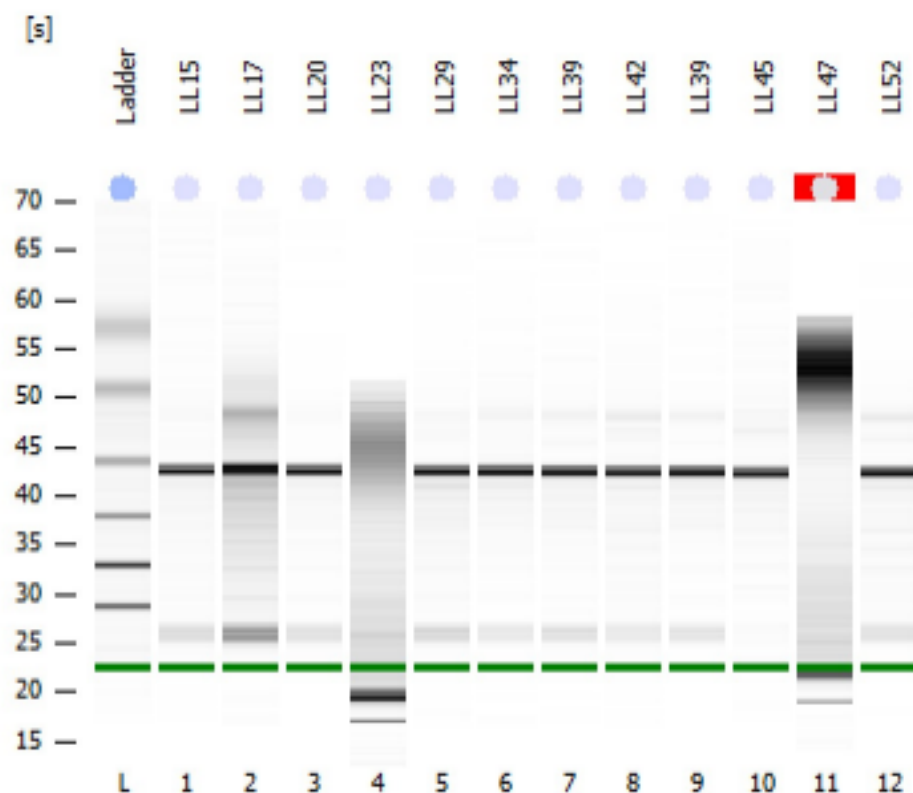


Figure 2.20 RNA quality profiles visualised via RNA nanochips by Agilent Technologies. Each lane represents total RNA from individual *Hermetia illucens* larvae,

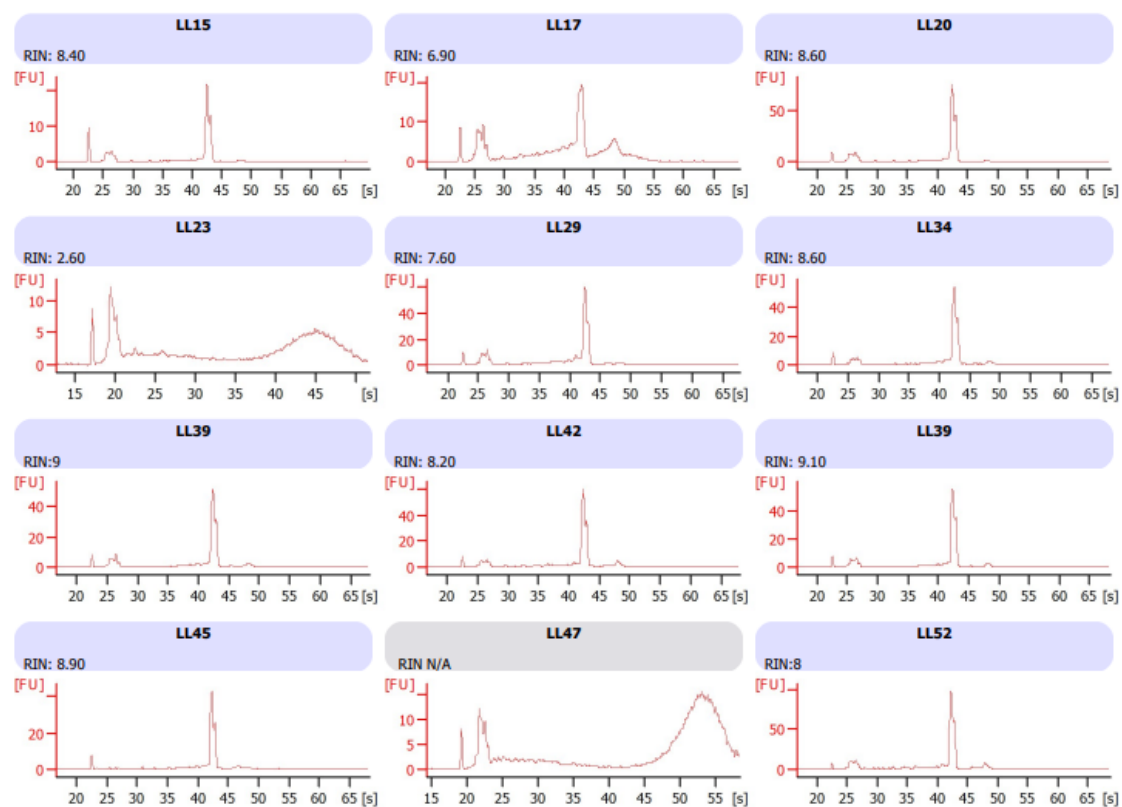


Figure 2.21 Electropherograms of *Hermetia illucens* RNA samples generated by the Agilent 2100 Bioanalyser. The x-axis represents migration time (s), while the y-axis represents fluorescence units (FU).

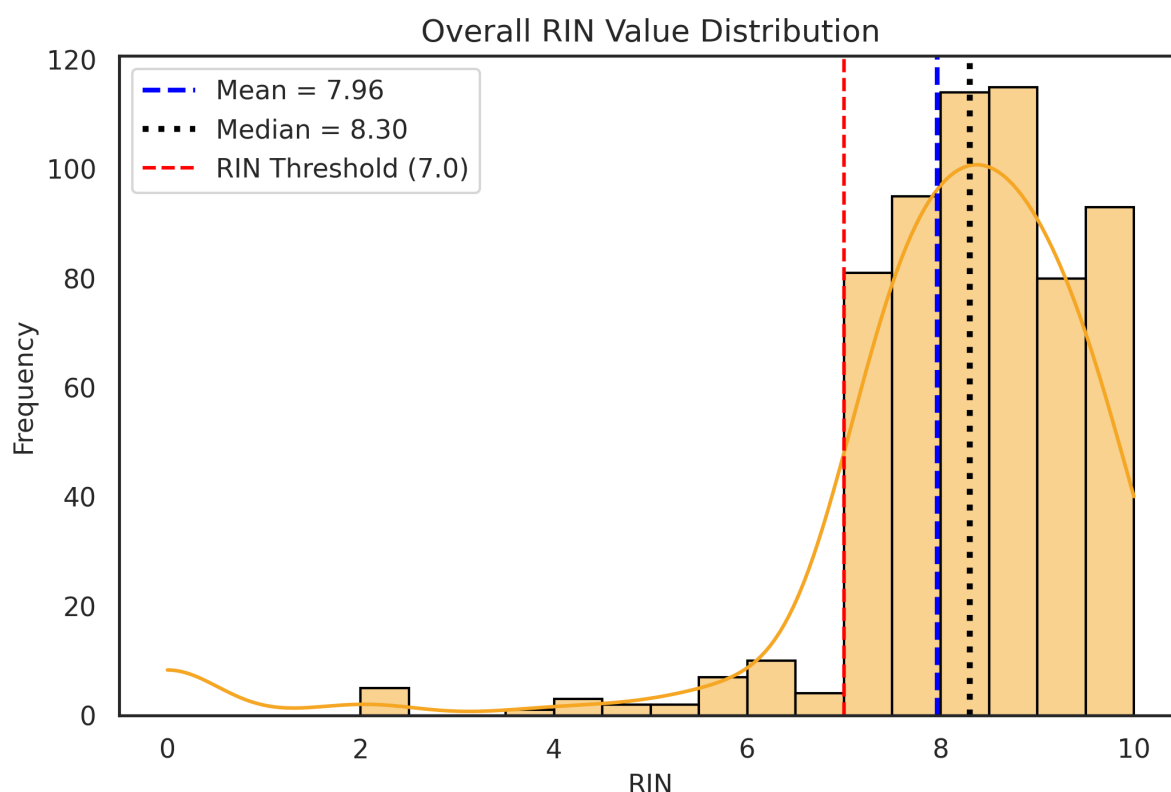


Figure 2.22 Overall distribution of RNA Integrity Number (RIN) values across all optimised extractions

2.26. Discussion

This chapter outlines the development and optimisation of a robust experimental platform for immune challenge and gene expression analysis in *Hermetia illucens* larvae. As a non-model insect with growing industrial relevance, the establishment of reproducible and biologically appropriate methodologies was essential to enable high-confidence transcriptomic and qPCR analysis. Therefore, the main goal was to address critical methodological gaps by optimising RNA extraction in BSFL – to enable reliable downstream molecular analyses.

Sub-optimal molecular workflows can systematically bias expression data, often without obvious warnings (Bustin, et al., 2010). Low or variably degraded RNA (e.g., poor RIN, low 260/230 from phenol/salt carry-over) can inflate Cq values and invert apparent fold-changes; co-extracted inhibitors (phenolics, polysaccharides, melanin, lipids, humic material from rearing substrates) alter polymerase kinetics and efficiency,

leading to artificial dose-responses or loss of significance even when biological signal is present (Schrader, Schielke, Ellerbroek, & Johne, 2012). In insects specifically, high endogenous RNase activity, chitinous/exoskeletal matrices, and microbial/food residues make clean extraction and inhibitor removal difficult, while the fat body's lipid content and haemolymph pigments further depress purity ratios (Fleige & Pfaffl, 2006) (Nascimento, et al., 2025). Given the lack of a one-size-fits-all protocol for insect tissues, we prioritised simple, cost-effective, and reproducible RNA workflows tailored to BSFL, coupled to strict QC, to safeguard the validity of downstream analysis.

Biological variability introduced before extraction can propagate into transcriptomic noise; for example, small shifts in rearing temperature can change larval developmental rate and immune profile (Wang, et al., 2025). Further to the above, injection protocols and suspension volumes were tested to minimise procedure-induced mortality and inconsistent transcriptional changes from wounding/osmotic stress.

To determine optimal rearing temperature and to avoid early pupation, and therefore, inconsistencies in our transcriptomic data, and potentially, qPCR analysis, we observed developmental progression at 25 and 28°C. Rearing larvae at 28°C reached both the prepupal and pupal phases more quickly and consistently; however, slower development at 25°C, was advantageous in experimental settings where prolonged larval stages were desired, such as for time-sensitive immune challenge studies. Maintaining larvae at 25°C allowed better control over premature pupation, reducing variability in developmental stage at the time of immune stimulation. This is consistent with reports that BSF larval development accelerates as temperature approaches the commonly cited 'rearing optimum' (~27–30°C), whereas cooler regimes (~24–25°C) prolong the larval window and delay pupation (Wang, et al., 2023).

Injection parameters were systematically tested to minimise handling-associated mortality. PBS was selected over physiological saline due to its improved compatibility with *H. illucens* haemolymph and enhanced survival rates post-injection. The third-to-last and penultimate metamere offered a reproducible and low-lethality injection route for introducing immune stimuli.

Immune stimulation experiments using pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (Poly I:C), allowed for immune stimulation without overwhelming larval viability. We evaluated injection volumes in 100 mg and 200 mg larvae. A 10 μ L volume was injected (~10% body mass in 100 mg larvae; ~5% in 200 mg larvae), and produced ~100% 24 h survival for 200 mg across PBS, LPS, and poly(I:C), but caused significant mortality for 100 mg larva most notably after LPS, with smaller decrease after poly(I:C) and (to a lesser extent) PBS. This is reflected in other studies in larva, where larger weights (~200-300 mg) were appropriate for 10 μ L volumes in disease challenge studies (Ignasiak & Maxwell, 2017). An injection volume of approximately 3-5% of larval body mass is a practical starting point for soft-bodied insect larvae; larger or smaller species may require adjustment.

Initial RNA extractions from both small (60–120 mg) and large (200 mg+) whole-body *Hermetia illucens* larvae, performed using the standard TRIzol protocol, revealed considerable variability in RNA quality and purity. Electrophoretic analysis showed frequent smearing and ambiguous banding. Spectrophotometric readings further corroborated these issues, with A260/230 ratios frequently falling below acceptable purity thresholds, suggestive of phenol, guanidine, or lipid contamination. Visual inspection during phase separation highlighted common extraction pitfalls: discoloured aqueous phases, carryover of lipid layers, and irregular pellet formation. These problems were exacerbated in larger larvae, likely due to increased fat and chitin content, which compromised phase separation and interfered with pellet resuspension (Figure 6B–C). This led to uneven RNA quantification and poor recovery in many standard TRIzol samples, raising concerns about downstream suitability for sensitive applications like qPCR or RNA-seq.

In many invertebrates (protostomes), the large subunit rRNA carries a taxon-specific “hidden break”, a thermosensitive nick in 28S rRNA that causes the molecule to split into two ~18S-sized fragments under heat or denaturing conditions (DeLeo, Pérez-Moreno, Vázquez-Miranda, & Bracken-Grissom, 2018). This break was first reported in *Hyalophora cecropia* (Applebaum, Ebstein, & Wyatt, 1966), and is caused by

denaturing (heating) RNA samples, and the separation of hydrogen bonds at 28Sa and 28Sb, which separates the two subunits. On standard denaturing gels or bioanalyzer traces, this collapse makes the 28S band disappear and the fragments co-migrate with 18S, producing an electrophoretic profile that mimics RNA degradation even when the RNA is intact (Ishikawa & Newburgh, 1972) (Ogino, Eda-Fujiwara, Fujiwara, & Ishikawa, 1990). Consequently, integrity metrics premised on clear 28S/18S resolution (e.g., 28S:18S ratios or mammal-calibrated RIN thresholds) can underestimate RNA quality in insects. This ‘break’ has been attributed to missing segments of nucleotides removed from the 28S precursors (e.g., *bombyx mori* ~30nt, *Sciara coprophila* ~19nt, and ~60nt in *Drosophila*) at a fixed position in ES5 and is associated with a recurring UAAU loop motif (Fujiwara & Ishikawa, 1986) (Ishikawa & Newburgh, 1972). The Black Soldier Fly (*Hermetia illucens*) also displays this hidden break in its 28S subunit, which is demonstrated by non-denatured and denatured samples but, in addition, like other studies involving insect RNA extraction, it proved difficult to maintain consistent, high-quality RNA for downstream analysis. RNA extractions in other insects such as *Thaumatotibia leucotreta*, *Thanatophilus micans*, *Plutella xylostella* and *Tenebrio molitor*, displayed issues across standard RNA extraction kits, highlighting the need for a species-specific approach in *Hermetia illucens*.

To overcome the challenges posed by the high protein, fat, and chitin content of *H. illucens* larvae, a modified BSFL TRIzol-based RNA extraction protocol was developed. This optimised method involved the longitudinal dissection of each larva into three sections to reduce tissue load per extraction and improve lysis efficiency. Homogenisation was performed using bead beating in TRI Reagent, followed by extended cold incubation steps to stabilise RNA and reduce enzymatic degradation.

Phase separation was achieved using bromochloropropane (BCP) alongside careful removal of lipid layers, helping minimise interphase disturbance during aqueous transfer. RNA was precipitated using a 1:1 volume of isopropanol, with samples gently inverted and incubated on ice to promote pellet formation and RNA precipitation solution was removed from the protocol. The RNA pellet was subsequently washed three times with 75% ethanol (instead of the standard one/two washes), which

significantly improved downstream purity metrics (A260/280 and A260/230), particularly by reducing residual phenol and salt contamination.

Following ethanol removal and air-drying, the RNA was resuspended in 100 µl of Milli-Q water and incubated at room temperature to enhance solubility. Due to the difficulty in pellet resuspension, samples were heated at 65°C for 5 minutes, without any issues to RNA integrity. Equal volumes from each larval section were then pooled into a single tube for long-term storage at -80 °C, preserving biological representation across the whole organism.

When tested against commercial column-based kits (e.g. RNeasy Mini), the optimised BSFL TRIzol protocol outperformed both the kit and the manufacturer's original instructions. It achieved higher yields (311 ng/µL vs. 120 ng/µL), better purity (A260/280 $\approx$ 2.05), and less sample dropout due to clogging or poor lysis (Figures 10–12). These findings strongly support the use of a refined TRIzol method for BSFL tissue, particularly when working with high-fat insect species where column capacity may be limiting. Assessment of ribosomal RNA profiles using the Agilent Bioanalyzer further validated these optimisations in addition to standardised gel electrophoresis procedures. Across 499 total samples, the average RIN was 7.96, and the median was 8.30, with most samples clustering between 7.5 and 9.5. This distribution confirmed that the amended protocol reliably produced RNA of sufficient integrity for transcriptomic analysis, even when canonical 28S:18S ratios were disrupted by the well-documented 28S hidden break.

While the amended BSFL TRIzol protocol introduces additional handling steps and necessitates rigorous quality control to ensure purity and integrity, it remains significantly more cost-effective than commercial column-based kits, particularly when applied to high-throughput or large-scale insect studies. The per-sample reagent cost of TRIzol-based extraction is substantially lower than that of silica column kits, making it more suitable for resource-limited or exploratory research. Thus, despite requiring greater input in terms of technical care and post-extraction validation, the refined BSFL

TRIzol workflow represents a robust and economically scalable option for extracting high-integrity RNA from complex insect tissues such as *H. illucens*.

Together, these findings demonstrate that RNA integrity in *H. illucens* also displays the hidden break discovered in other protostomes, and is highly sensitive to extraction conditions, especially in whole-body protocols where lipid content is high. The stepwise refinements introduced here- dissection/incubation, careful phase separation, removal of RNA precipitation, multiple ethanol washes, and thermal-assisted pellet resuspension were critical to achieving consistently high-quality RNA. Importantly, these optimisations ensured that only samples meeting stringent purity thresholds (A260/280 between 1.9–2.2 and A260/230 between 2.0–2.4) proceeded to pooling and cDNA synthesis, enhancing the reliability of all downstream molecular analyses.

Chapter 3. Identification of Canonical Innate Immune Genes in the Black Soldier Fly Larvae (BSFL) Transcriptome

3.1. Introduction

As interest grows surrounding The Black Soldier Fly (*Hermetia illucens*) as a sustainable source of proteins and lipids for animal feed (Kaczoe, Bulak, Proc-Pietrycha, Kirichenko-Babko, & Bieganski, 2022), (Lomonaco, et al., 2024) as a tool for organic-waste bioconversion (Rehman, et al., 2022), and as a biotechnology platform (Zhu, et al., 2019), (Zabulione, Salaseviciene, Makstutiene, & Sarkinas, 2023), so does the need to understand the underlying biological mechanisms that govern its physiology, immunity, and development. While morphological and ecological studies (Bonelli, et al., 2020), (Eke, et al., 2023), (Chia Y. S., et al., 2018) (Hu, et al., 2023), (Mannucci, et al., 2024) have provided valuable insights into the life cycle and environmental adaptability of *H. illucens*, the molecular and genetic basis of many key traits remains poorly understood.

A high-quality chromosome-level reference genome for the Black Soldier Fly (*Hermetia illucens*) has now been made available by Generalovic and colleagues. The G3 assembly, generated using PacBio long reads, 10X Genomics linked reads, and Hi-C scaffolding, spans ~1.01 Gb with 99.75% of sequence placed on seven chromosomes (six autosomes plus X), contig N50 $\approx$ 16.0 mB, scaffold N50 $\approx$ 180.5 mB, and BUSCO completeness $\approx$ 98.6%. Roughly 67% of the genome is repetitive, and ~16,478 protein-coding genes were annotated using the BRAKER2 pipeline.

Transcriptomics, the study of actively expressed genes through RNA sequencing, provides a powerful approach to bridge this knowledge gap (Wang, Gerstein, & Snyder, 2009). By revealing the functional output of the genome at specific life stages or under specific biological conditions, transcriptomics can uncover the genes and regulatory pathways that drive BSF performance, adaptation, and, importantly for this study, immune responses. This is particularly relevant for the discovery of antimicrobial peptides (AMPs), stress-response genes, and immune signalling components that are of interest not only in basic insect biology, but also in applied contexts such as pathogen response profiling and host-microbe interactions.

Although several studies have begun to explore the BSF transcriptome across different tissues and development stages, the available data remains fragmented, with limited focus on whole-body transcriptomes and immune-related functional annotation. As BSF continues to be selectively bred and optimised for industrial applications, detailed transcriptomic maps will be essential for understanding strain-specific traits and guiding targeted improvements. In this chapter, we explore the role of transcriptomics in advancing our understanding of the *H. illucens* immune system.

Despite increasing commercial interest in *Hermetia illucens*, knowledge of its innate immune system remains fragmented compared with established insect models such as *Drosophila melanogaster*. While individual antimicrobial peptides and immune-associated genes have been reported, a comprehensive understanding of the signalling pathways and molecular networks underlying pathogen recognition and immune activation remains incomplete. This lack of annotation limits the interpretation of immune challenge experiments and hinders the development of molecular tools for studying host-pathogen interactions in BSFL.

To address this knowledge gap, transcriptomic approaches provide a powerful strategy for characterising immune systems in non-model organisms. Through genome-guided annotation and orthology-based comparisons, it is possible to identify conserved components of canonical immune pathways, including Toll, IMD, JNK, and JAK/STAT signalling cascades, which coordinate pathogen recognition, intracellular signal transduction, and effector responses in insects.

Therefore, the primary aim of this chapter was to generate a transcriptome-derived immune gene resource for *H. illucens* through functional annotation and classification of immune-related transcripts. Emphasis was placed on identifying genes involved in pathogen perception, signal integration, and immune response, thereby establishing a foundation for the antimicrobial peptide expression analyses presented in Chapter 4 and the host-pathogen interaction studies described in Chapter 5. The resulting immune gene catalogue provides a valuable resource for future functional studies and

contributes to the development of molecular tools for investigating immunity, disease resilience, and host–microbe interactions in BSFL.

3.2. Background in Transcriptomics

Transcriptomics is the comprehensive study of all RNA transcripts produced by the genome under specific conditions, providing insight into which genes are active, to what extent, and in which contexts (Stark, Grzelak, & Hadfield, 2019). Unlike genomics, which reveals the blueprint of potential gene function, transcriptomics captures the dynamic and functional output of gene expression at a given moment. This makes it a powerful tool for understanding biology, particularly in response to environmental cues, developmental changes, or disease.

Modern transcriptomic studies are mostly conducted using RNA sequencing (RNA-Seq), a high-throughput method that enables the quantification of gene expression across the entire transcriptome. RNA-Seq has replaced older techniques such as microarrays due to its greater sensitivity, dynamic range, and the ability to detect novel transcripts (Head, et al., 2014).

To enrich for protein-coding mRNA transcripts, polyadenylated (polyA) RNA is often selectively isolated from total RNA. This method enhances the sequencing focus on messenger RNAs (mRNAs), which are typically polyadenylated at their 3' ends, thereby excluding non-coding RNAs and ribosomal RNAs that may otherwise dominate the dataset. This polyA enrichment approach is particularly relevant when the objective is to explore genes that encode functional proteins, such as antimicrobial peptides or immune-related receptors.

3.3. Current Transcriptomic Research in *Hermetia illucens*: Insights and Advances

Transcriptomic studies on *Hermetia illucens* have grown steadily over the last decade, reflecting the increasing scientific and commercial interest in this species. RNA sequencing (RNA-Seq), meta-transcriptomics, and targeted gene expression analysis have been applied to explore a wide range of traits, including metabolism, immunity, microbiome interactions, stress responses, development and sensory biology. The following transcriptomic studies provide an overview of the key advances to date.

Zhu et al. (2019) conducted de novo transcriptome sequencing to investigate fat accumulation during larval development. They sequenced 24 libraries across 12 BSF stages on Illumina HiSeq X Ten and built their de novo transcriptome with Trinity (>70 unigenes; N50 > 1.7kb), then quantified DE on the assembled transcripts. The authors assembled 70,475 unigenes and identified 14,597 differentially expressed transcripts, then mapped expression across the pathways supplying acetyl-CoA, building fatty acids, and assembling TAG. Here, the authors focus on the core carbohydrate-lipid axis that feeds TAG synthesis, profiling representative steps in glycolysis (e.g., HK, PFK), the citrate shuttle (CS, ACLY), fatty-acid synthesis/activation (ACC, FASN, ACS family), and TAG assembly (AGPATs, PLPPs, DGAT1). Expression is broadly higher at both early larval and late pupal windows, but differential signals indicate early TAG turnover versus late net TAG accumulation, matching the measured lipid peak at early prepupa. Overall, the dataset pinpoints control nodes across the pathway and offers tractable targets for metabolic engineering and selection aimed at boosting total fat (Zhu, et al., 2019).

Another study explored the impact of microbiota on BSF gene expression by utilising library preparation of poly(A)-enrichment (NEBNext Ultra II Directional) and RNA-seq (Illumina NovaSeq 6000) using axenic (germ-free) larvae compared to larvae reared under normal, non-sterile conditions. In a germ-free (axenic) larva versus conventional comparison of *Hermetia illucens*, the microbiota exerted clear, gene-level effects across immunity, digestion, and metabolism. Conventional larvae consistently up-regulated immune effectors, notably Defensin-A, Dipterocin-D and Lysozyme, together

with chitin-associated genes (Acidic mammalian chitinase, *cbd-1*), consistent with microbiota-primed humoral defence and cuticle/gut barrier turnover. Axenic larvae showed up-regulation of PGRP-LB, a regulator of the IMD pathway, and several digestive proteases (trypsin's, chymotrypsin-like), plus TCTP; furthermore, they reported a general dampening of transcriptome expression from this group across multiple pathways (Auger, Bouslama, Deschamps, & Derome, 2023).

Vogel et al. generated a de novo larval transcriptome of *H. illucens* and profiled diet-dependent immune expression. Libraries were prepared and sequenced by GATC Biotech on an Illumina HiSeq 2500: paired-end 2 x 100 bp for 14 whole-larva diet libraries and single-end 1 x 100bp for mixed diet/tissue libraries, yielding ~30-40 million reads per sample. RNA-seq reads were combined for de novo assembly in CLC Genomics Workbench v8.1, producing a reference 'backbone' of 25,197 contigs (minimum 250 bp; contig N50 = 1,330 bp; maximum contig 23,709 bp). Screening the assembly revealed an expanded repertoire of antimicrobial peptides (AMPs) in BSF. They reported 53 putative AMP genes spanning major insect families – 26 defensins, 10 dipterocins, 7 cecropins, 6 attacins, and 4 knottin-like peptides, together with multiple lysozyme transcripts. Beyond AMPs, they also found other immune components, such as PGRPs, GNBP, and phenoloxidases associated with melanisation. Crucially, AMP expression was diet-sensitive: substrates differing in composition and microbial load elicited distinct AMP profiles, and these transcriptional shifts were accompanied by substrate-specific antibacterial activity (Vogel, Muller, Heckel, Gutzeit, & Vilcinskas, 2018).

Shah et al. profiled the temporal transcriptomic response of five-day-old *Hermetia illucens* larvae to clean wounding versus Gram-negative infection with *Pseudomonas protegens* Pf-5. Larvae were assigned to Untreated, PBS (wounding control), or Pf-5 (~5x10³ CFU) injections and sampled at 2, 6, and 13 h (n=5 biological replicates per treatment x time; two larvae pooled per replicate), yielding 45 TruSeq Stranded Total RNA libraries sequenced on NovaSeq PE150 (~19.2 M reads/sample; GC » 38.5%). Reads (Q>35) were aligned with STAR to the *H. illucens* reference (Generalovic T. , et al., 2021), achieving ~78% unique mapping; 15,196 genes were detected and filtered to

11,900 for DESeq2 analysis. Wounding produced an early, transient immune response of upregulated recognition/signalling genes at 2 hours (e.g., PGRP-SA, Relish) and effectors (cecropins, defensins, attacins), which largely subsided at 13h, followed by the up-regulation of metabolism/growth genes (e.g., trypsin's, chymotrypsin's). In contrast, infection triggered a delayed yet expansive response: only 6 genes up at 2 h, rising to 347 up-regulated/341 down-regulated at 6 h and 818 upregulate/678 downregulated at 13 h, with sustained induction of IMD-axis signatures (Relish, multiple PGRPs) and a broad AMP burst, including many cecropin paralogues (23) and several defensins (5). Concurrent, coherent down-regulation of cuticle, OBP99a/b, cytochrome P450s, lipases, and other metabolic/cell-cycle processes support a pronounced immunity-metabolism trade-off during bacterial challenge. Collectively, the study delineates a brief wound-healing phase versus a robust, sustained antibacterial program and nominates AMP and recognition genes as practical biomarkers for BSFL health monitoring (Shah, Maistrou, Willemsen, Van Loon, & Dicke, 2024).

Zhang et al. compare swill- versus pig-manure diets using tissue-resolved RNA-seq and untargeted LC-MS metabolomics to ask how substrate shapes BSF larval physiology. Day-8 larvae were dissected into cuticle, fat body, gut, Malpighian tubules and haemolymph (pools of 10 per sample). mRNA libraries (ABclonal kit) were sequenced on Illumina NovaSeq 6000, yielding $\sim 7.58 \times 10^8$ high-quality reads in total ($\sim 6.32 \times 10^7$ per library; Q30 > 94%) with >80% mapping to the reference (unnamed); reads were aligned with HISAT2, counted by featureCounts, FPKM-normalized, and differential expression was called by DESeq2. Data are deposited under BioProject PRJNA1159383. Swill-fed larvae showed a broader up-shift in gene expression, with DEGs enriched for core metabolism, carbon metabolism, glycolysis/TCA, amino-acid biosynthesis, redox processes, and KEGG over-representation across metabolic and thermogenesis pathways. Pig-manure larvae, by contrast, displayed more gene down-regulation overall and selective induction of stress/defence programs, including immune-related categories (e.g., antimicrobial peptide/immune defence annotations). Metabolomics detected 2,598 features; swill increased organic acids, amino acids and triglycerides consistent with elevated anabolic flux, whereas pig manure increased stress-linked signatures such as short-chain fatty acids and antioxidants. Together, the multi-omics

readout supports a substrate-dependent physiology in which nutritionally diverse swill promotes growth and energy metabolism, while microbially and chemically challenging pig manure biases larvae toward stress responses and constrained metabolic output (Zhang, et al., 2024).

Another group compared a balanced larval diet (SD) with a nutritionally poor fruit-vegetable mix (VMD) to ask how the *H. illucens* midgut compensates for low-quality substrates. This was achieved by a complementary midgut de novo transcriptome (6 libraries; ~516M reads; 27,102 transcripts after filtering; N50 ~2.1 kb; BUSCO ~97% eukaryote/~94% Arthropoda complete). This captured 1,910 differentially expressed transcripts, with enrichment of proteolysis, transport, and metabolic processes in VMD and coordinated down-regulation of ferritin genes. Together, the study highlights that BSFL may maintain performance on poor substrate by ramping proteolysis/uptake while dealing with starch and lipid digestion, aided by a structural remodelling of the posterior midgut (Bonelli, et al., 2020).

Hull et al. profiled whole-body RNA-seq to determine two things: (i) whether an extra generation in mass-rearing (F2>F3) shifts expression, and (ii) whether deliberate selection (a line bred for larger larvae) adds its own signature. Libraries were stranded Illumina RNA-seq, short-read, and reads were mapped to the chromosome-level BSF reference genome (Generalovic T. , et al., 2021). Counts were generated per gene, and differential expression was called with standard pipelines, using significance and fold-change thresholds. They found a generational effect with ~900 differentially expressed genes (DEGs), enriched for metabolism, development, and defence/immune response. For the selection regime (non-selected vs selected line): ~200 DEGs were found, largely centred around metabolism (consistent with selection for greater larval mass). Detox/Immune genes (notably cytochrome P450s such as CYP6A1, CYP9F2) were higher in F2 than in F3 and negatively correlated with larval mass, which could be interpreted as an immune/defence vs growth trade-off. Developmental signs also shifted: NITO (sex/eye development in flies) was higher in F2 and negatively associated with mass, whereas Rumi (Notch O-glucosyltransferase) increased across generations and was highest in the selected line, positively associated with mass – suggesting faster

development/greater metabolic throughput under selection. The selected line showed upregulation of CRYL1-like (LOC119661460), IDH1 (LOC119648135), FGGY kinase (LOC119649137), trehalose transporter Tret1-1 (LOC119655675) and CG7200, all of which, were positively associated with larval mass, supporting enhanced energy production/ROS management within the strain-specific larvae. These findings underscore the importance of developing strain-specific genomic and transcriptomic resources to guide future breeding programs, as reliance on generic or wild-type reference data may overlook important functional differences emerging through domestication (Hull, Alexander, Bruneaux, & Christoffels, 2022).

DeRaedt and colleagues sequenced small RNAs from five life stages (egg, L5 larva, pupa, adult (male), adult (female) n=3 per stage; 15 libraries) prepared with Illumina TruSeq Small RNA, sequenced single-end (mean read length 51 nt) to ~ 10.6-16.4 M raw reads per library, mapped to the “BSF genome” (assembly not specified). They identified 192 BSF miRNAs: 168 conserved (arthropod orthologs) and 24 BSF-unique. Among the BSF-unique miRNAs, 10/24 were stage-restricted (e.g., eggs: hil-miR-m, -p, -r, -s, -u; pupa: hil-miR-ac; adult (female): hil-miR-h; adults of both sexes: hil-miR-a, -b, -y); notably, no unique miRNA was larva-only. Canonical developmental miRNAs showed expected stage trends (e.g., let-7 peaking in pupae; bantam higher in larvae), underscoring roles in development, stress responses, and immune modulation. To infer potential regulatory targets, the authors mapped 22 conserved BSF miRNAs to 56 experimentally validated targets in *Drosophila*, speaking to the conserved component of these networks. The 24 BSF-unique miRNAs support species-specific post-translational regulation that now warrants functional validation in BSF (DeRaedt, 2022).

In a dual omics study, Liu et al. selectively bred BSF larvae for tolerance at 16°C (T16) and 40°C (T40) and profiled both transcriptomes and proteomes of the bred lines vs temperature-matched controls (C16/C40). They ran short-read RNA-seq for differential expression, plus full-length transcriptome sequencing in the T16 line, and LC-MS/MS proteomics for protein-level changes. After breeding at 16°C for 11 generations, they found several carbohydrate metabolic pathways were enriched, suggesting BSFL likely utilise carbohydrate metabolic pathways to cope with cold stress. Multiple AKR1B1-like

(aldose reductase) transcripts, the rate-limiting gateway to the polyol pathway (glucose > sorbitol > fructose), indicating a shift of glucose flux toward polyol/cryoprotectant production under thermal stress. Enrichment was also noted at 16°C for autophagy-animal genes, heat shock proteins and immune genes within the signalling pathways: Toll, IMD, chemokine, and RIG-I-like, suggesting these too play a role in counteracting thermal stress. A total of 27,414 novel transcripts were retrieved at 16°C, with most pointing to novel transcripts in metabolic and immunomodulatory mechanisms (Feng, et al., 2024).

This study sequenced adult antennal transcriptomes of *Hermetia illucens* to catalogue chemosensory genes and profile their expression. Libraries (TruSeq) were run on an Illumina HiSeq 2500 (paired end). Reads were assembled de novo with Trinity, yielding 118.6M raw reads and 119.99 M clean reads, producing 70,142 unigenes. From this data, the authors identified 27 odorant-binding proteins (OBPs), 5 chemosensory proteins (CSPs), 70 odorant receptors (ORs), 25 ionotropic receptors (IRs), 10 gustatory receptors (GRs), and 2 sensory neuron membrane proteins (SNMPs) (Xu, Wu, Zeng, & An, 2020).

3.4. Current Reference Genomes of *Hermetia illucens*

There are now a few reference genomes that underpin *Hermetia illucens* research. The first widely used draft, by Zhan and colleagues, was built from high-coverage Illumina short reads and delivered a ~1.1Gb assembly with ~1.7Mb scaffold N50 and 16,770 protein-coding genes, near-complete by BUSCO but comparatively fragmented (Zhan, et al., 2020). A subsequent chromosome-level resource (iHerIll2.2.curated.20191125) from Generalovic et al., combining PacBio long reads, 10x linked reads and Hi-C to produce a 1.01 Gb assembly with contig and scaffold N50s of ~ 16 Mb and ~ 180.5 Mb, respectively, placing ~99.75% of sequence onto seven chromosomes (six autosomes plus X) with BUSCO ~98.6% and 16,478 genes. This has since been the main gene-context reference and is also served by NCBI/Ensembl (Generalovic T. , et al., 2021). An independent chromosome-scale build from Costagli et al. used PacBio plus Omni-C,

yielding an 888.6 Mb assembly with ~162.2 Mb scaffold N50 and ~89% BUSCO; importantly, Omni-C enabled analysis of topologically associated domains, but overall completeness is lower than iHerIII (Costagli, Abenaim, Rosini, Conti, & Giovannoni, 2024). Finally, a Japanese-strain preprint (Takeda et al., 2025) contributes whole-genome sequence data with an annotated gene set integrated with RNA-seq; but currently, as a non-peer-reviewed resource as of August 2025 (Takeda, et al., 2025).

3.5. Curating an Immune Gene Database for Black Soldier Fly Research

As *Hermetia illucens* industries look to scale up and develop their scientific applications, there is a clear need for standardised, systematic resources to study and manage BSFL immunity (and beyond). A curated immune-gene database, grounded in transcriptomic evidence and *Drosophila melanogaster* orthology, would help provide the foundation for health management protocols (surveillance biomarkers, early warning thresholds, and response profiles) and for genomic selection programmes aimed at disease resistance/tolerance. While *Drosophila melanogaster* has been the reference model for studying insect immune pathways, future research will need to account for species-specific differences relative to *Drosophila* as well as strain-level variation within *H. illucens* (arising from domestication, diet, and geographic origin). Given the growing pressures on mass-reared populations, including potential disease outbreaks and microbial imbalances, the development of an immune gene catalogue will assist in monitoring key immunological components.

Transcriptomic profiling could help identify key regulators such as pattern recognition receptors, signalling intermediates, and effector molecules, many of which are already annotated through comparative approaches to model organisms like *Drosophila melanogaster*. By mapping and classifying these immune-relevant transcripts, researchers and industry partners alike can begin to integrate immunogenomic data into breeding, biosecurity, and functional studies.

In this study, we present a filtered and annotated transcriptome of the BSF strain EVE through Better Origin Ltd, Cambridge, with a focus on identifying and classifying transcripts with putative immune function. We used a pooled approach comprising whole-body (WB) RNA to capture a comprehensive transcriptomic profile encompassing gut, fat body, haemocytes, and cuticular tissues, all of which are relevant to immune function and stress response. Furthermore, including three different rearing substrates allows us to capture a broader view of immune pathways within BSFL.

This foundational dataset not only supports further immunological research in BSF but also provides a scalable template for gene curation as the species becomes increasingly central to circular bioeconomy systems.

3.6. Methodology

3.7. Ethical Considerations

All experimental procedures involving *Hermetia illucens* larvae were reviewed and approved by the Animal Welfare and Ethical Review Body (AWERB) under the University of Stirling, reference number AWERB 2023 12442 11402. All rearing, handling, and sampling procedures were conducted with due consideration for animal welfare, minimising unnecessary stress and maintaining consistent husbandry conditions across all treatment groups.

3.8. Study organisms and strains

We used a commercial *H. illucens* production strain ('EVE') supplied by Better Origin (UK). EVE is maintained under standard industrial conditions and was selected to represent a widely used production genotype.

3.9. Sample Collection and RNA Extraction

Whole-body larvae of *Hermetia illucens* were reared on three different substrates: Control diet (Gainesville), Spent Brewers Grains, and Cow Manure. For each dietary treatment, six brood splits were sampled (n=6 per treatment), resulting in a total of 18 biological replicates. RNA was extracted from each of these samples following an amended TRIzol Reagent protocol:

3.10. Homogenisation

Each dissected section was placed into a labelled screw-cape tube containing 1mL of TRI Reagent. Samples were incubated on ice for 30 minutes before homogenization using a bead beater for 30 seconds then incubated on ice for 5 minutes.

3.11. Phase Separation

Samples were then centrifuged at 20,000 x g for 15 minutes 4°C, after which, 600 µL of each sample was transferred to a new tube with 100 µL of BCP (avoiding any lipid layer upon transfer) and incubated on ice for 15 minutes. After another centrifugation step at 20,000 x g for 15 minutes at 4°C, 200 µL of the aqueous phase was transferred to a fresh tube, taking care not to disturb the interphase.

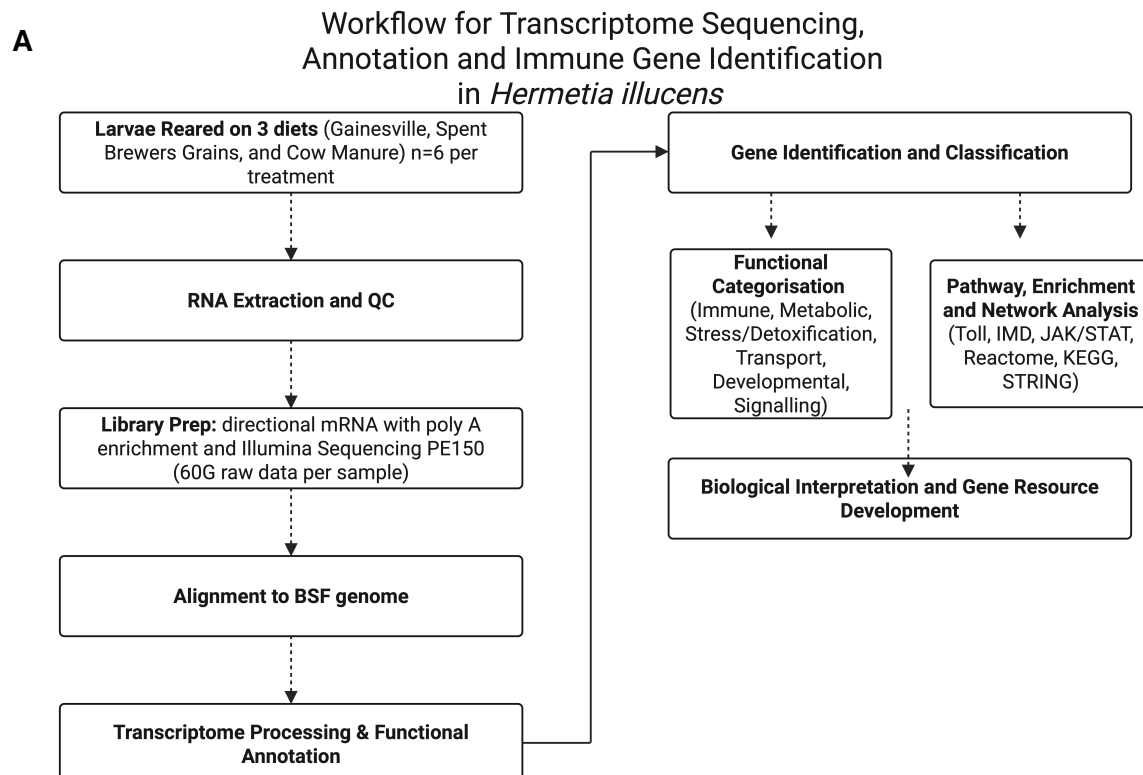
3.12. RNA precipitation

200µL of 100% isopropanol (1:1) was added to the aqueous phase, followed by gentle inversion (4-6 times) and incubation on ice for 10 minutes at 4°C. Samples were centrifuged at 10,000 x g for 10 minutes at 4°C, forming a gel-like RNA pellet. The pellet was washed three times with 1mL of 75% ethanol, flicked to lift the pellet, and centrifuged at 20,000 x g for 5 minutes at room temperature. Residual ethanol was removed by air-drying, and RNA was resuspended in 100 µL of Milli-Q water and incubated at room temperature for 30 minutes to facilitate resuspension.

RNA from the 18 whole-body larvae of strain EVE was pooled into a single library at a final concentration of 400ng/μL prior to sequencing.

3.13. RNA Quality Assurance and Sequencing

The BSFL transcriptome was achieved through NOVOGENE for Directional mRNA library preparation (poly A enrichment) and Illumina Sequencing PE150 (60G raw data per sample). To ensure the extracted RNA was ready for sequencing, RNA concentrations and Integrity were measured using an Agilent 2100 Bioanalyzer with RNA nanochips through Agilent Technologies (No. 5067-1511). Pool A had an Integrity Number (RIN) of 7.0 (Figure 3.1), confirming that RNA quality was suitable for poly-A-enriched, strand-specific library preparation, and an appropriate RIN score for insect whole-body RNA.



B

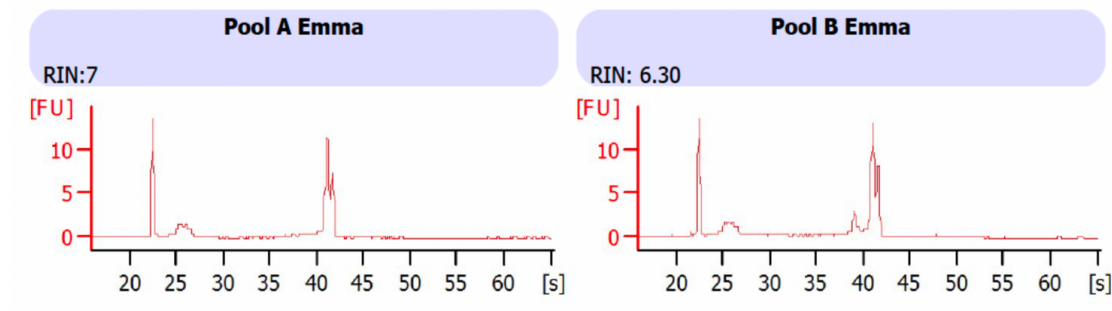


Figure 3.1 (A) Methodology workflow (Created in <https://BioRender.com>) and **(B)** Agilent 2100 Bioanalyzer electropherograms of pooled *Hermetia illucens* RNA samples prepared for library construction. The x-axis represents migration time (s), while the y-axis represents fluorescence units (FU). RNA Integrity Number (RIN) values are shown for each sample and indicate RNA quality prior to library preparation.

Sequencing data was processed using the NF-core/rna-seq pipeline (Figure 3.2), implemented via Netflow, on a high-performance computing cluster. The pipeline was run with default parameters, using the green route: STAR + SALMON workflow for splice-aware genome alignment and transcript-level quantification.

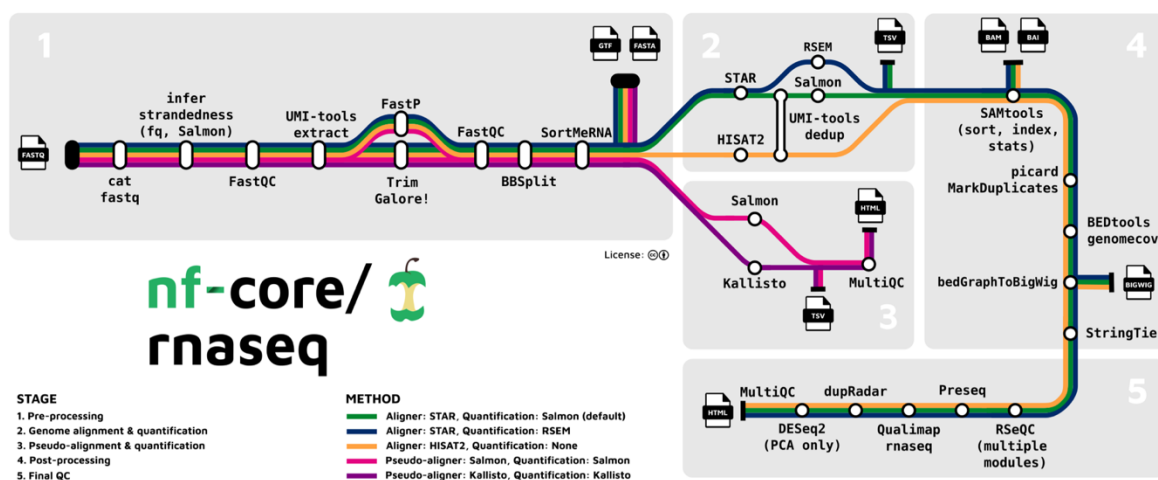


Figure 3.2 Schematic representation of the *nf-core/rnaseq* pipeline

3.14. Pre-processing

Before alignment to the Black Soldier fly genome, Raw FASTQ files were assessed for quality using FastQC. Trim Galore! cleared low-quality bases and adapters, while SortMeRNA filtered out Ribosomal RNA. These processed reads were validated again using FastQC, and all metrics were compiled using MultiQC.

3.15. Alignment and Quantification

Reads were aligned to the *Hermetia illucens* reference genome, assembled by Tom Genorlovic (Generalovic T. , et al., 2021) RefSeq: GCA_905115235.1, a chromosome-level assembly. STAR, a splice-aware aligner optimised for RNA-seq, was used for genome-guided alignment. Transcript abundance was quantified using Salmon, in alignment-based mode, maintaining strand specificity throughout.

3.16. Post-processing and Quality Control

To ensure proper, efficient clean-up of the data for downstream analysis, SAMtools and Picard were used to sort and index BAM files while also marking duplicates. Furthermore, Bedtools, bedGraphToBigWig, and StringTie were used to calculate genome coverage and generate transcript structures, while quality metrics, duplication rates, and read distribution were assessed using Qualimap, dupRadar, and RSeQC. Once completed, a further QC step was generated via MultiQC.

3.17. Transcriptome Filtering and Functional Annotation

A total of 68607 transcript sequences were initially obtained from the full transcriptome assembly, generated using a genome-guided approach. Functional annotation was performed using BlastP searches against the UniProt Swiss-Prot database to identify putative protein-coding genes and assign molecular function.

Out of the total transcripts, 38,620 were retained based on the presence of a valid `sprot_Top_BLASTP_hit` identifier, indicating a confident functional match. These formed the filtered transcriptome set used for further downstream analysis.

3.18. Species Distribution of Functional Hits

To characterise the origin of the functional annotations assigned during BLASTP analysis, the species source of the top UniProt matches was examined; species information was extracted from the corresponding UniProt accession and summarised by organism.

3.19. Immune Gene Identification and Classification

To identify transcripts associated with immune function, a custom Python script was developed and executed within a Jupyter Notebook environment. This script systematically screened all annotated genes from the BLASTP-filtered transcriptome against a comprehensive set of curated immune-related keywords, derived from UniProt functional annotations, known immune gene symbols, and canonical components of insect immunity pathways (e.g., Toll, IMD, JNK, JAK-STAT).

Each gene's BLAST-derived name was converted to lowercase to ensure consistent string matching. Using a keyword-to-category dictionary, genes were scanned and assigned to one of the three immune functional categories:

1. **Perception:** Genes involved in pathogen detection (e.g., PGRPs, Tolls, Scavenger Receptors).
2. **Integration:** Genes mediating signal transduction and regulation (e.g., MyD88, TRAF, MAPK cascade).
3. **Response:** Genes involved in immune effector execution (e.g., antimicrobial peptides, apoptosis mediators, phenoloxidase cascade components).

The classification pipeline was implemented using the following Python packages: pandas for loading, filtering, and managing large annotation tables, matplotlib.pyplot to visualise the number of genes identified per immune category and collections.Counter to count occurrences of each immune category in the final dataset.

Where multiple transcripts shared identical UniProt hits, Power Query in Microsoft Excel was used to retain only the highest-confidence transcripts, based on alignment (Query/Hit span), BLAST E-value, and percentage identity. This helped eliminate redundancy and improve annotation specificity.

3.20. Gene set enrichment and visualisation

Gene lists from the transcriptome analyses were submitted to g:Profiler (g:GOSt) as a single-query analysis. The organism was set to *Drosophila melanogaster*. Enrichment was queried against GO Biological Process (GO:BP), GO Molecular Function (GO:MF), GO Cellular Component (GO:CC), and KEGG using g:Profiler's default statistical domain scope. g:Profiler's adjusted p-values (q) were used for inference; results were interpreted against a threshold of $q \leq 0.05$, while full ranked tables were retained. CSV files were processed in Python 3.12.8 using imports "textwrap", "pathlib", "numpy", "pandas", "matplotlib", matplotlib.pyplot". Column names were normalised to lowercase, and the following fields were retained: source (database), term_name, term_id, adjusted_p_value (q), term_size, and intersection_size. Non-data header artefacts and rows with missing or invalid q values were removed. A plotting score was calculated as $-\log_{10}(q)$. For KEGG visualisations, non-informative umbrella categories (KEGG:01100 Metabolic pathways and KEGG:00000 KEGG root term) were excluded to avoid generic signals. Figures were generated as 300 dpi PNGs. Terms were ranked by $-\log_{10}(q)$ and the top N terms were displayed as a single column with colour intensity proportional to $-\log_{10}(q)$; bar graphs and dot plots were generated using the same score/ratio with dynamically wrapped labels and margins to prevent clipping.

3.21. Reactome Pathway Enrichment Analysis

To identify pathway-level functional enrichment, transcriptome-derived protein annotations were analysed using the Reactome Pathway Database (<https://reactome.org>). Protein-coding transcripts were first annotated via BLASTP against the UniProt/Swiss-Prot database, and the top hits were used to assign gene identifiers. These UniProt entries were then converted to *Drosophila melanogaster* FlyBase gene identifiers (FBgn format) using the g:Profiler (g:Convert) tool, ensuring compatibility with the Reactome analysis platform.

The resulting list of FlyBase gene IDs was submitted to Reactome's overrepresentation analysis tool, specifying *Drosophila melanogaster* (NCBI Taxon ID: 7227) as the species of interest. Reactome assessed enrichment by comparing the submitted gene list to curated pathway annotations using a hypergeometric test. The output included both raw p-values and Benjamini-Hochberg-corrected false discovery rates (FDR) for multiple testing.

A total of 1,485 unique genes from the input list were successfully mapped to at least one Reactome pathway and included in the enrichment analysis. Pathways were ranked by raw p-value, and results were interpreted in the context of known biological processes relevant to immunity, stress response, and cellular regulation. While no pathways passed FDR correction thresholds, the most enriched pathways (by raw p-value) were explored for biological relevance and reported in the results.

3.22. Protein-protein interaction and pathway enrichment analysis (STRING)

Protein-protein interaction (PPI) analysis was performed using the STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins; version 12.0) to investigate functional relationships among proteins identified within the annotated *Hermetia illucens* transcriptome. As STRING currently has limited annotation for *H. illucens*, proteins were first assigned to their closest *Drosophila melanogaster* orthologues

during the annotation process, and these orthologues were subsequently used for STRING analysis.

Networks were generated using all available evidence channels, including experimentally validated interactions, curated databases, gene neighbourhood, gene fusion, gene co-occurrence, co-expression, text mining and homology. The default medium confidence interaction score (0.400) was applied. STRING was used to calculate protein-protein interaction enrichment statistics, identify significantly enriched biological pathways and functional modules, and generate interaction network visualisations.

To investigate broader functional organisation, enrichment analyses were performed within STRING using Gene Ontology (Biological Process, Molecular Function and Cellular Component), KEGG, Reactome and WikiPathways databases. Significantly enriched terms were exported from STRING as tab-delimited files for downstream analysis.

To identify conserved signalling hubs across enriched pathways, the complete enrichment output ('All enriched terms') was analysed using custom Python scripts (Python version 3.12). Gene occurrence was quantified by counting the number of enriched KEGG, Reactome and WikiPathways terms in which each gene appeared. Genes were subsequently ranked according to pathway frequency to identify proteins repeatedly represented across multiple signalling pathways, providing an indication of potential pathway hubs and conserved signalling components. Summary tables and graphical visualisations were generated using the Pandas and Matplotlib Python libraries.

3.23. Results

3.24. Species Distribution

Of the total 38,620 filtered hits, a total of 20,922 hits (54%) were matched to well-annotated model organisms, with the highest representation being from *Drosophila melanogaster* (15,394 hits) (Table 3.1; Figure 3.3), reflecting the high-quality annotation of dipteran pathways. Additional top species included *Homo sapiens* (5,803), *Mus Musculus* (4138) and *Bos Taurus* (1605), likely due to conserved core genes shared across taxa. Hits from insect species such as *Aedes aegypti* (430), *Anopheles gambiae* (310), *Bombyx mori* (161), further support the biological relevance of immune-related hits in invertebrates. The dominance of *Drosophila melanogaster* is notable, as this species serves as a long-standing reference model for insect immunity, with extensive experimental evidence supporting Toll, IMD, and JAK/STAT pathway characterisation (Lemaitre & Hoffmann, 2007) (Buchon, Silverman, & Cherry, 2014).

Table 3.1 Species distribution of top BLASTP hits from the filtered transcriptome of *Hermetia illucens*

DROME	DROSOPHILA MELANOGASTER	15,394
HUMAN	<i>Homo sapiens</i>	5,803
MOUSE	<i>Mus musculus</i>	4,138
BOVIN	<i>Bos taurus</i>	1,605
RAT	<i>Rattus norvegicus</i>	1,331
DANRE	<i>Danio rerio</i>	895
XENLA	<i>Xenopus laevis</i>	843
CAEEL	<i>Caenorhabditis elegans</i>	678
CHICK	<i>Gallus gallus</i>	665
XENTR	<i>Xenopus tropicalis</i>	463
AEDAE	<i>Aedes aegypti</i>	430
DROMA	<i>Drosophila mauritiana</i>	425
DROPS	<i>Drosophila pseudoobscura</i>	341
ANOGA	<i>Anopheles gambiae</i>	310

PONAB	<i>Pongo abelii</i>	234
NASVI	<i>Nasonia vitripennis</i>	220
POPJA	<i>Populus japonica</i>	216
ARATH	<i>Arabidopsis thaliana</i>	185
PIG	<i>Sus scrofa</i>	183
BOMMO	<i>Bombyx mori</i>	161
OTHER	All other species (≤ 160 hits)	4,099

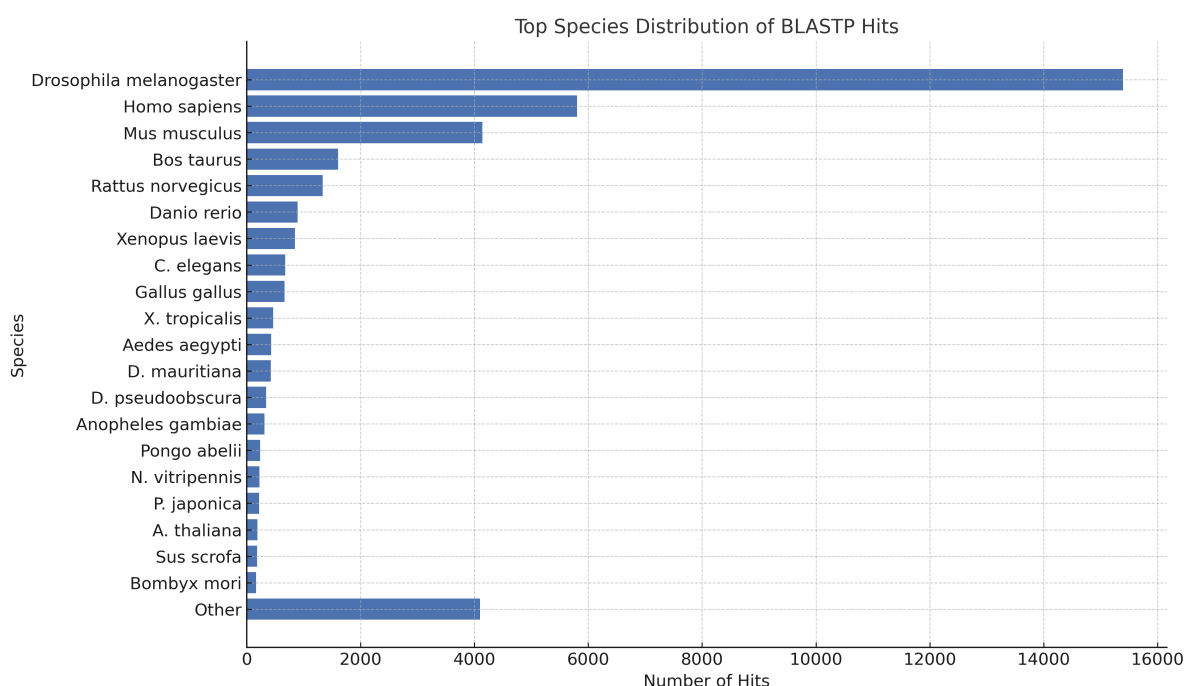


Figure 3.3 Bar plot highlighting species distribution from filtered BLASTP hits

3.25. Overview of Identified Immune Genes: Perception, Integration, and Response

From the total filtered BLASTP hits, 191 genes were identified (Appendix) with predicted immune-related functions based on homology and functional keyword screening, particularly across Toll, IMD, and JAK-STAT pathways (ID%: 23.6-100%, E_Value: 0.0-1.76e-06). Mapping these conserved immune signalling cascades is crucial for elucidating how *Hermetia illucens* responds to microbial threat signalling at a molecular level. These pathways govern the detection of pathogens, through the activation of pattern recognition receptors (PRRs), and the communication of

downstream modules via intracellular signals, to ultimately, execute an immune response such as antimicrobial peptide (AMP) production, apoptosis, and melanisation.

The Toll pathway primarily regulates the expression of AMPs like defensins and Drosomycins in response to Gram-positive bacteria and fungi, while the IMD pathway controls responses to Gram-negative bacteria, including the production of Attacins and Diptericins. JNK and JAK-STAT contribute to stress responses, immune modulation and tissue regeneration. By tracing the presence and expression of components within these pathways, we can begin to assess the immune competence of *H. illucens* under different environmental or microbial exposures, identify targets for enhancing AMP yield, and improve biotechnological applications in waste bioconversion, feed safety, and disease resilience.

These genes were cross-referenced against well-annotated databases, with the final list representing a diverse panel of innate immune genes relevant to host defence, signalling, and effectors.

Immune genes were functionally grouped into three broad categories:

Perception: Encompassing 37 genes, this category includes pattern recognition receptors (PRRs), such as peptidoglycan recognition proteins (PGRPs) and Toll-like receptors, involved in sensing microbial or danger signals.

Integration: Key signalling transduction pathways were discovered, with a total of 102 genes captured from keyword screening. These included key adaptors such as MyD88 and IMD, kinases such as Pelle and TAK1, and transcriptional regulators like Relish and Dorsal.

Response: 46 genes were captured from this group; among these were antimicrobial peptides, apoptosis mediators (e.g., Dredd, Caspases), and effector enzymes involved in melanisation and oxidative defence.

The distribution highlights a strong representation of signal transduction genes (Figure 3.4), which is expected given the conserved and modular nature of immune signalling cascades. The relatively lower number of PRRs may reflect the limited annotation of species-specific receptors outside of model organisms.

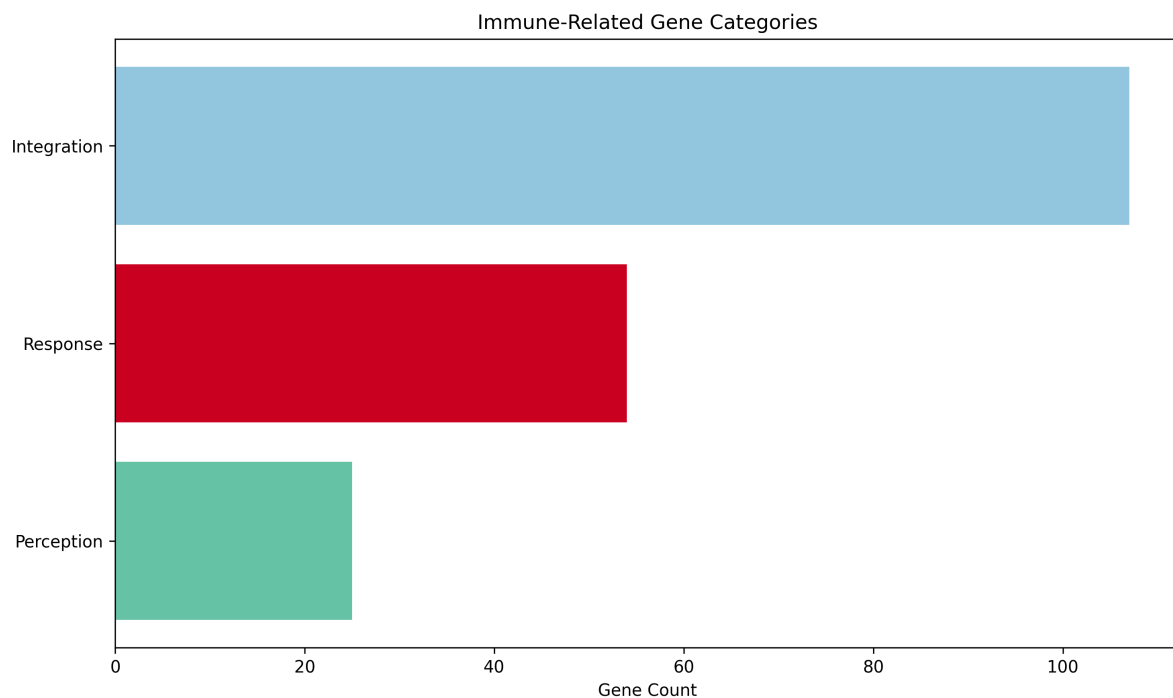


Figure 3.4 Number of immune genes per functional category

3.26. Pathway-Specific Groupings: Key innate pathways in Black Soldier Fly Larvae

Within the classified genes, canonical components of several key innate immune pathways were detected:

3.27. Toll Pathway:

The toll signalling pathway is a hallmark of insect innate immunity and is particularly well-characterised in Diptera such as *Drosophila melanogaster*. Given the high degree of conservation observed in core immune signalling pathways across Dipteran species, including *Aedes aegypti* (Ramirez & Dimopoulos, 2010), *Anopheles gambiae* (Osta,

Christophides, Vlachou, & Kafatos, 2004), (Clayton, Dong, & Dimopoulos, 2013), and *Musca domestica* (Zhao, Wang, Li, & Gai, 2020), it is highly probable that the Toll pathway is functionally conserved in *Hermetia illucens*. Originally discovered in *Drosophila melanogaster* for its role in embryonic development (Anderson, Jurgens, & Nusslein-Volhard, 1985), (Hashimoto, Hudson, & Anderson, 1988) and later established as a key regulator of immune responses, particularly against fungal pathogens and Gram-positive bacteria (Lemaitre, Nicolas, Michaut, Reichhart, & Hoffmann, 1996). Activation of the Toll pathway is typically triggered through recognition of lysine-type peptidoglycan or fungal β -glucans by extracellular pattern recognition receptors (PRRs), including PGRP-SA and GNBP. These initiate a serine protease cascade (involving modSP, Grass and SPE), which ultimately cleaves the cytokine-like ligand Spätzle into its active form. Cleaved Spätzle binds to the Toll Receptor, initiating an intracellular signalling cascade via the adaptor proteins MyD88, Tube, and the kinase Pelle. This leads to phosphorylation and degradation of the I κ B-like inhibitor Cactus, thereby releasing NF- κ B transcription factor Dorsal to translocate into the nucleus and activate immune effector genes, including antimicrobial peptides (AMPs) such as Drosomycin, and Defensins (Table 3.2; Appendix Table 5).

Table 3.2 Toll pathway candidate genes identified in *Hermetia illucens* transcriptome via BLASTP annotation

Gene ID	Common Name	E-Value	% Identity	Span
pgpsa_drome	PGRP-SA	1.52e-60	53.3	Q:21-187,H:37-203
modsp_drome	modSP	1.12e-14	29.1	Q:1-155,H:302-462
grass_drome	Grass	3.34e-11	37	Q:55-140,H:116-212
Spz_drome	Spz	3.74e-34	31.2	Q:101-326,H:93-326
toll_drome	Toll Receptor	1.31e-74	38.3	Q:464-842,H:551-942
toll6_drome	Toll Receptor 6	1.18e-59	73.2	Q:46-224,H:509-687
toll7_drome	Toll Receptor 7	0	72.1	Q:39-1310,H:58-1364
toll8_drome	Toll Receptor 8	0	73.3	Q:49-1286,H:4-1220
myd88_mouse	Myd88	9.63e-28	31.1	Q:27-306,H:20-289
tube_drome	Tube	3.71e-27	38.5	Q:2-176,H:27-213
kpel_drome	Pelle	1.72e-101	45.4	Q:3-391,H:92-491
cact_drome	Cactus	1.3e-87	52	Q:153-490,H:140-493
dors_drome	Dorsal	1.45e-159	44	Q:1-701,H:222-997

3.28. IMD pathway:

The Immune Deficiency (IMD) pathway is one of the central pillars of insect innate immunity, particularly responsible for mediating defence against Gram-negative bacterial infections (and in some cases, Gram-positive). It is analogous in part to the Tumour Necrosis Factor Receptor (TNFR) pathway in mammals, highlighting the evolutionary conservation of immune signalling cascades.

In *Drosophila melanogaster*, IMD pathway activation is initiated by the recognition of diaminopimelic acid (DAP)-type peptidoglycan, a component of Gram-negative bacterial cell walls, by membrane-bound pattern recognition receptors such as PGRP-LC or PGRP-LE. Ligand binding leads to receptor oligomerisation and downstream recruitment of IMD, which serves as a key adaptor protein. This, in turn, interacts with FADD and the caspase DREDD, resulting in the cleavage and activation of the NF- κ B transcription factor Relish. Activated Relish translocate to the nucleus, where it promotes the transcription of antimicrobial peptides (AMPs), including Attacins, Dipterocins, and Cecropins.

In the *Hermetia illucens* transcriptome, all components of the IMD pathway were found based on BLASTP annotations and keyword screening (Table 3.3; Figure 3.5; Appendix Table 6).

Table 3.3 *IMD pathway candidate genes identified in Hermetia illucens transcriptome via BLASTP annotation*

Gene ID	Common Name	E-Value	% Identity	Span
pgple_drome	PGRP-LE	1.86e-78	45.1	Q:30–361, H:29–337
imd_drome	IMD	5.06e-36	43.5	Q:96–252, H:96–265
fadd_drops	FADD	1.46e-29	32	Q:15–241, H:1–240
casp8_drome	DREDD	7.54e-90	37.8	Q:13–498, H:10–491
nfkb1_drome	Rel	1.13e-122	38.2	Q:76–724, H:151–803

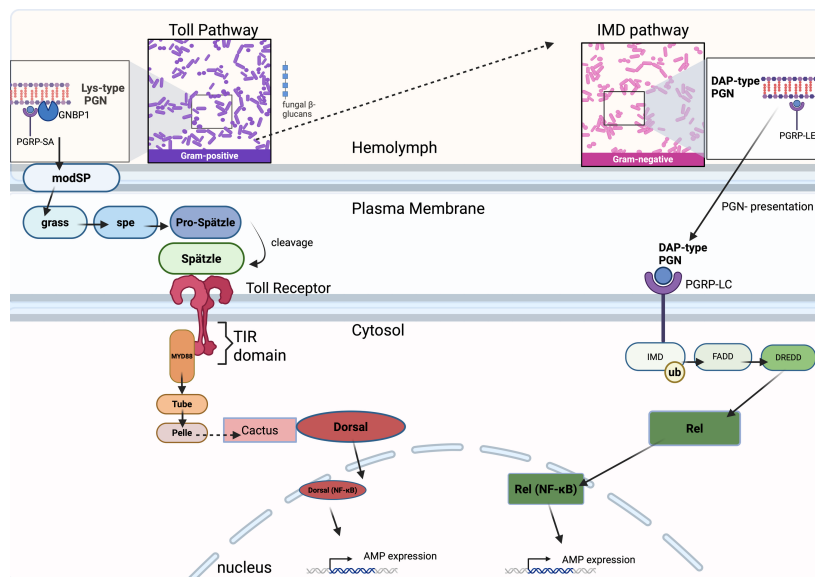


Figure 3.5 Schematic representation of the canonical Toll and IMD pathways in Dipteran insects, based primarily on *Drosophila melanogaster*. The toll pathway (left) is typically activated in response to fungal or Gram-positive bacterial infections via recognition of pathogen-associated molecular patterns (PAMPs) such as β -glucans or LYS-type peptidoglycan (PGN). Pattern recognition receptors (e.g., PGRP-SA, GNBPs) initiate a proteolytic cascade (modSP > Grass > SPE), leading to cleavage of pro-Spätzle and subsequent activation of the Toll receptor. Intracellularly, a conserved signalling module comprising MyD88, Tube, and Pelle activates the NF- κ B transcription factor Dorsal via Cactus degradation, promoting antimicrobial peptide (AMP) expression. The IMD pathway (right) responds to Gram-negative bacteria through PGRP-LC recognition of DAP-type peptidoglycan (PGN), triggering downstream components including IMD, FADD, and DREDD, culminating in Relish (NF- κ B) activation and AMP gene expression. Although the Toll pathway has not yet been functionally characterised in *Hermetia illucens*, several orthologous components have been identified in transcriptomic and genomic datasets, suggesting conservation of core pathway architecture within Diptera. Diagram created in <https://BioRender.com>.

3.29. JAK/STAT:

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is a compact, cytokine-responsive cascade that couples extracellular danger cues to context-specific gene expression (Arbouzova & Zeidler, 2006). In *Drosophila*, the pathway was defined through the discovery of its core components: the JAK kinase Hopscotch (*Hop*) (Binari & Perrimon, 1994); the STAT transcription factor *stat92E* (Marelle) (Hou, Melnick, & Perrimon, 1996); the secreted cytokine ligands Unpaired (Upd) (Harrison, Binari, Gilman, & Perrimon, 1998); and the type I cytokine receptor Domeless (*Dome*) (Brown, Hu, & Hombría, 2001).

In insects, parasitic challenge, viral infection, enteric Gram-negative bacteria, and sterile stress/wounding induce Upd3 ($\pm$ Upd2) in target tissues including haemocytes,

fat body, gut epithelium and imaginal discs which activates Dome/Hop signalling and STAT92E-dependent transcription (Agaisse, Petersen, Boutros, Mathey-Prevot, & Perrimon, 2003), (Souza-Neto, Sim, & Dimopoulos, 2009), (Buchon, Broderick, Poidevin, & Lemaitre, 2009), (Yang, Kronhamn, Ekstrom, Korkut, & Hultmark, 2015), (Katsuyama, Comoglio, Seimiya, Cabuy, & Paro, 2015). Ligand binding to Dome triggers Hop-mediated phosphorylation, STAT92E dimerisation and nuclear import, where target genes are induced, and the negative regulator SOCS36E is transcribed to provide feedback control (Bina & Zeidler, 2013), (Ekas, et al., 2010), (Stec, Vidal, & Zeidler, 2013).

These homology searches recover a complete JAK-STAT toolkit in *H. illucens*. The core signalling nodes: Domeless (cytokine receptor), Hopscotch/JAK (tyrosine kinase) and STAT, all return strong, domain-spanning matches, with STAT and JAK showing especially high conservation typical of effector kinases/transcription factors, and Domeless aligning across most of its extracellular and intracellular regions (as is common for large receptors) (Table 3.4, Figure 3.6; Appendix Table 7)

Although the identification and classification of immune-associated genes provide insight into the molecular repertoire present within the annotated transcriptome, interpretation at the individual gene level does not fully capture the broader functional organisation of the dataset. Functional enrichment analyses were therefore undertaken to identify biological processes and signalling pathways that were statistically over-represented within the annotated transcriptome. Gene Ontology (GO), KEGG and Reactome enrichment analyses were subsequently performed to provide systems-level insight into the functional landscape of the transcriptome before investigating protein-protein interaction networks and signalling hub architecture.

Table 3.4 JAK/STAT pathway candidate genes identified in *Hermetia illucens* transcriptome via BLASTP annotation

Gene ID	Common Name	E-Value	% Identity	Span
dome_drome	Domeless/Dome	1.74e-53	24.2	Q:38-948,H:22-975
jak_drome	Hopscotch/JAK	1.02e-264	39.9	Q:4-1056,H:46-1151
stat_drome	Stat	1.11e-103	62.7	Q:1-260,H:435-695
socs5_bovin	Socs/Socs36e	9.87e-73	72.3	Q:399-564,H:359-524
ptps_drome	Ptps1	7.02e-72	64.6	Q:4-165,H:5-168
rbp2_drome	Sumo	2.63e-45	29.8	Q:134-402,H:2420-2709
Pias_human	Pias	4.4e-115	49.1	Q:1-392,H:10-405
nu301_drome	Eb(x)	7.66e-157	47.7	Q:23-667,H:901-1515
ken1_culqu	Ken	1.1e-121	51.3	Q:2-615,H:4-653

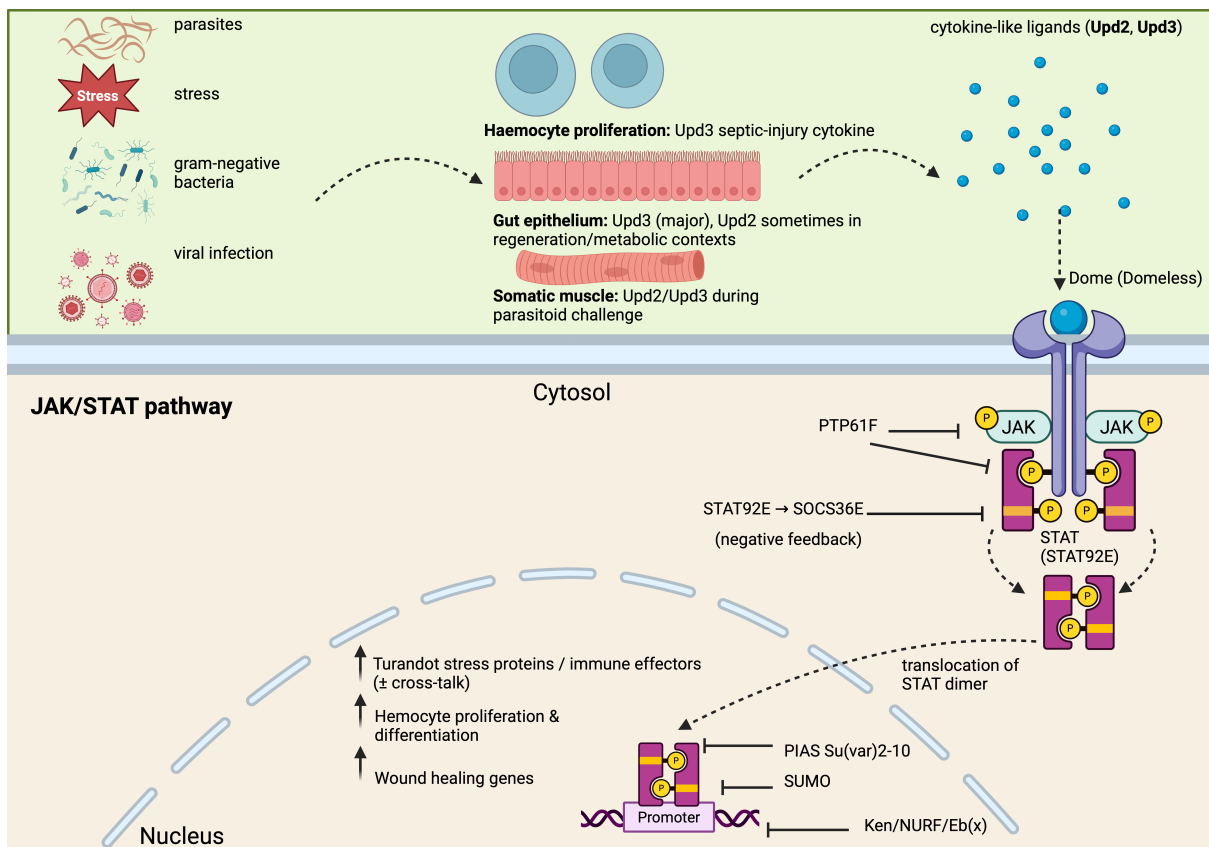


Figure 3.6 Overview of the *Drosophila* JAK/STAT signalling pathway and its activation by immune and stress stimuli. Various stimuli, including parasitic infection, physiological stress, Gram-negative bacterial infection, and viral infection, trigger the production of cytokine-like ligands Unpaired 2 (Upd2) and Unpaired 3 (Upd3) in specific tissues. Upd3 is the major ligand produced by the gut epithelium during septic injury, while Upd2 is sometimes expressed in regeneration or metabolic contexts. In somatic muscle, Upd2/Upd3 are induced during parasitoid challenge, and in haemocytes, Upd3 acts as a key cytokine promoting proliferation. These ligands bind to the transmembrane receptor Domeless (Dome), activating the associated Janus kinase (JAK) Hopscotch. This leads to phosphorylation of the receptor and subsequent recruitment and phosphorylation of the signal transducer and activator of transcription protein Stat92E (STAT). Activated STAT forms dimers, translocates to the nucleus, and binds to promoter regions to induce transcription of target genes, including Turandot stress proteins, immune effectors, haemocyte proliferation/differentiation genes, and wound-healing genes. Pathway activity is regulated by negative feedback via Suppressor of Cytokine Signalling 36E (SOCS36E) induced by STAT92E, as well as dephosphorylation by PTP61F.

Additional regulation occurs through protein inhibitors such as PIAS, SUMOylation, and chromatin modifiers including Ken, NURF, and Eb(x). Diagram created in <https://BioRender.com>.

3.30. Gene Ontology and KEGG pathway enrichment analysis

To ensure reliable functional annotation, all transcript sequences were subjected to BLASTP searches against the UniProt Swiss-Prot database. A stringent filtering criterion was applied, retaining only transcripts with valid `sprot_Top_BLASTP_hit` entries (Figure 3.7). This yielded a total of 38,620 high-confidence alignments, which formed the foundation for downstream immune gene curation and enrichment analyses.

All retained hits demonstrated highly significant similarity (Table 3.5), with E-values ranging from 0.0 to 9.84e-06 (median: 1.34e-76), reflecting robust statistical confidence in the homology assignments. The minimum E-value of 0.0 corresponds to perfect or near-perfect matches, while the maximum value was 9.84e-06.

Percent identity values across hits ranged from 19.0% to 100.0%, with a median identity of 49.1%, indicating that over half of the alignments displayed moderate-to-high sequence similarity. The interquartile range (IQR) for identity was 38.5%–63.4%, suggesting consistent homology across most matches. Additional descriptive statistics are provided in Table 3.5.

Importantly, 100% of the retained alignments passed conventional stringency cutoffs, supporting the validity of inferred gene functions. These parameters collectively underscore the high confidence and utility of the filtered transcriptome for functional annotation, immune gene discovery, and comparative analyses.

Transcript Retention After BLASTP Filtering (n=68,606)

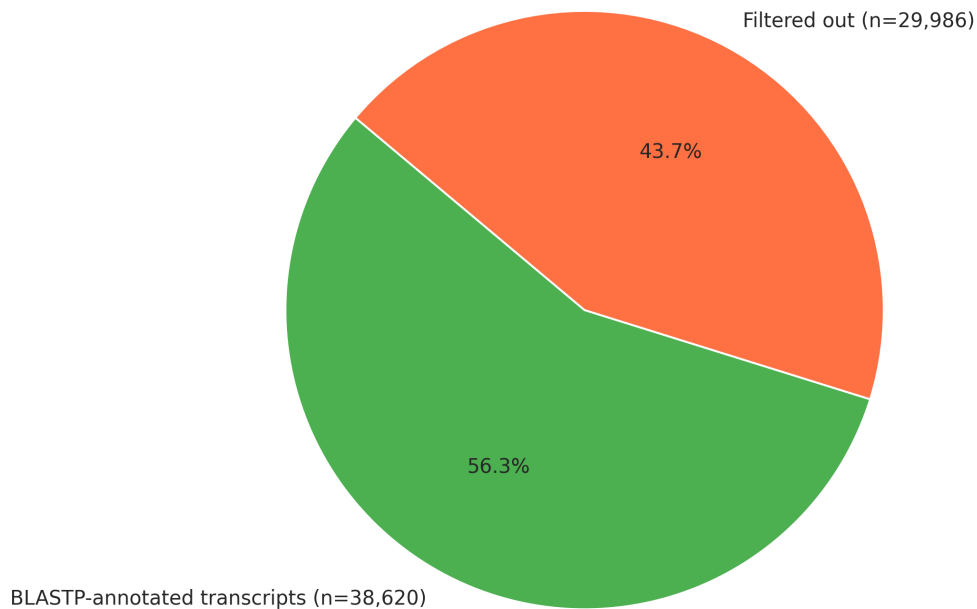


Figure 3.7 BLASTP-annotated transcript retention after filtering

Table 3.5 Summary statistics of BLASTP alignment quality for the filtered *Hermetia illucens* transcriptome

Metric	Minimum	25th Percentile	Median	75th Percentile	Maximum	Mean	Std. Dev.
% Identity	19.0%	38.5%	49.1%	63.4%	100.0%	52.0%	17.3
E-value	0.0	1.44e-150	1.34e-76	1.47e-35	9.84e-06	3.68e-08	4.24e-07

3.31. GO: Biological Process and Molecular Function (GO:BP; GO:MF)

Enrichment analysis of the Biological Process category revealed strong overrepresentation of terms associated with developmental morphogenesis, metabolic regulation, and pigmentation processes (Figure 3.8, left). Highly significant terms included renal tubule morphogenesis (GO:0061333), regionalisation (GO:0003002), and tube morphogenesis (GO:0035239), indicating transcriptional activity related to tissue patterning and organ development. Multiple pigmentation-associated processes, including pigment metabolic process (GO:0042440) and developmental pigmentation

(GO:0048066), were also enriched, which may reflect cuticular and ocular pigment production.

Metabolic processes were well represented, particularly those linked to amino acid, pyrimidine-containing compound, and nucleobase metabolism. The enrichment of digestive system development (GO:0055123) and epithelial cell proliferation (GO:0050673) suggests active tissue turnover and gut epithelial regeneration, processes likely beneficial for BSFL reared on microbially rich substrates.

The Molecular Function category highlighted enrichment for catalytic and binding activities (Figure 3.8, right). Notably, acid-thiol ligase activity (GO:0016879), purine nucleotide transmembrane transporter activity (GO:0015216), and DNA binding, bending (GO:0008301) suggest active regulation of DNA structure and nucleotide metabolism.

The presence of hydrolase activity, acting on carbon-nitrogen bonds (GO:0016812) and aromatic amino acid transmembrane transporter activity (GO:0015173) points towards enhanced metabolic flexibility. Binding-related functions, including vitamin binding (GO:0019842) and pigment binding (GO:0031409), support metabolic pathways identified in GO:BP.

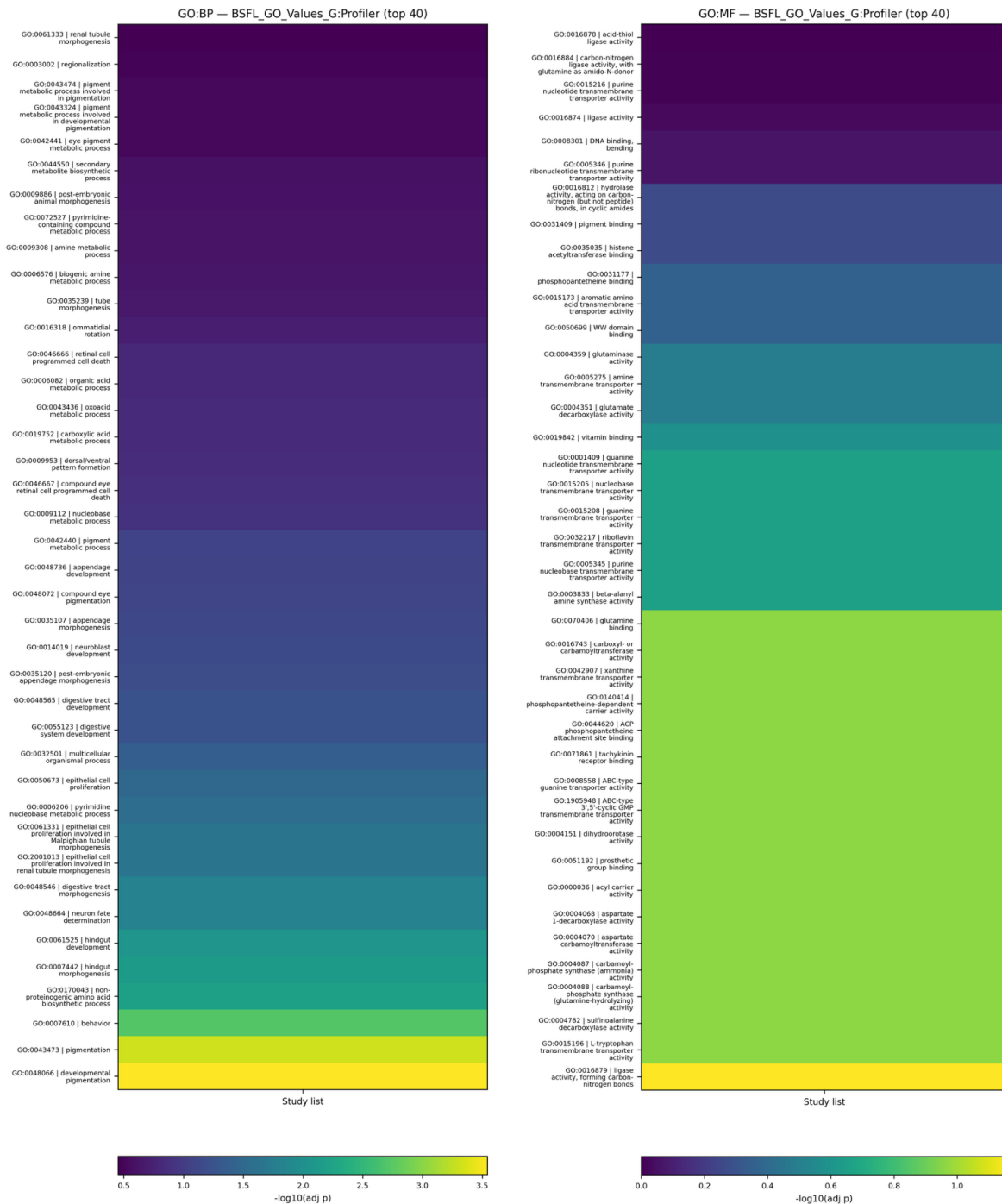


Figure 3.8 Gene Ontology enrichment analysis of Black Soldier Fly Larvae (BSFL) transcriptome data. The top 40 significantly enriched Biological Process (GO:BP, left) and Molecular Function (GO:MF, right) terms were identified using *g:Profiler* analysis of BSFL annotated genes. Colour intensity represents adjusted *p*-values transformed as $-\log_{10}(\text{adj } p)$, with darker shades indicating higher significance. GO terms are ranked along the vertical axis. Heatmaps made on Python.

3.32. GO: Cellular Component (GO:CC) and KEGG pathways

Enrichment within the Cellular Component category (Figure 3.9, left) demonstrated overrepresentation of membrane-bound organelles and trafficking complexes, including Golgi apparatus subcompartment (GO:0098791), endoplasmic reticulum (GO:0012505), and endosome membrane (GO:0010008). Such enrichment reflects a transcriptional investment in protein processing, modification, and vesicular transport, mechanisms central to secretion of digestive enzymes and immune effectors. The enrichment of clathrin-coated vesicle membrane (GO:0030665) and AP-type membrane coat adaptor complex (GO:0030119) indicates active endocytosis and intracellular trafficking, consistent with gut epithelial turnover and immune surveillance functions. The identification of pigment granule (GO:0048770) again links to pigmentation pathways, further supporting findings from GO:BP analysis.

KEGG pathway enrichment analysis (Figure 3.9, right) revealed overrepresentation of biosynthesis of cofactors (KEGG:01240), pyrimidine metabolism (KEGG:00240), and lysosome (KEGG:04142) pathways. These findings suggest high metabolic activity and enhanced degradative capacity, potentially linked to nutrient recycling and pathogen degradation within the gut (Perera et al., 2016).

The enrichment of Notch signalling (KEGG:04330) aligns with its established roles in insect gut regeneration and immune regulation (Micchelli and Perrimon, 2006), while pantothenate and CoA biosynthesis (KEGG:00770) and beta-alanine metabolism (KEGG:00410) indicate investment in coenzyme production and amino acid catabolism—processes important for energy homeostasis. The detection of dorso-ventral axis formation (KEGG:04320) underscores the involvement of developmental pathways, which may also be co-opted during tissue repair.

3.33. Pathway-Level Enrichment (Reactome)

To complement GO term enrichment and provide pathway-level resolution, Reactome analysis was performed using BLASTP-annotated transcripts mapped to *Drosophila melanogaster* FlyBase gene identifiers (2,680 hits). Of the submitted transcriptome-derived gene set, 1,485 (55.4%) unique FlyBase gene identifiers (FBgns) were successfully mapped to at least one Reactome pathway for *Drosophila melanogaster*, enabling overrepresentation analysis and functional interpretation.

Among these, 102 unique genes involved in the top 20 pathways (ranked by raw p-value) revealed functional enrichment in a variety of cellular processes (Table 3.6; Figure 3.10), most notably:

- Nuclear pore complex disassembly and nuclear envelope breakdown, processes critical to cell division, stress responses, and immune regulation.
- SUMOylation-related pathways, including those targeting chromatin and DNA replication proteins, suggesting widespread post-translational regulation linked to transcriptional control and DNA repair.
- RNA transport mechanisms, such as SLBP-dependent and intronless transcript export, which may reflect shifts in translational control during immune or stress challenge.
- Hippo/Warts signalling and ERK activation pathways, both of which are known to interface with immune modulation and apoptosis in insects.
- Pathways related to cell cycle checkpoints and replication initiation, consistent with previous observations of proliferative and homeostatic processes being engaged in response to environmental stimuli.

While no pathways passed FDR correction due to the breadth of comparisons, raw p-values remain informative in this exploratory context. The diversity of pathway matches reflects the broad transcriptional scope of the dataset and supports the presence of genes involved in stress adaptation, immune signalling, and fundamental cellular maintenance.

While enrichment analyses identify significantly represented biological processes and pathways, they do not describe how the encoded proteins interact with one another. Therefore, STRING protein-protein interaction analysis was performed to identify highly connected modules and signalling networks.

Table 3.6 Top 20 enriched Reactome pathways identified from BLAST-P annotated transcripts converted to FlyBase gene IDs

#	Pathway Name	Genes Matched	Genes in Pathway	p-value	FDR
1	Nuclear Pore Complex (NPC) Disassembly	26	30	2.45e-02	7.97e-01
2	Nuclear Envelope Breakdown	30	38	4.73e-02	7.97e-01
3	SUMOylation of SUMOylation proteins	24	30	6.20e-02	7.97e-01
4	Postmitotic NPC Reformation	17	20	6.80e-02	7.97e-01
5	Regulation of Glucokinase by GK Regulatory Protein	25	32	7.12e-02	7.97e-01
6	DNA Replication Initiation	8	8	8.87e-02	7.97e-01
7	DS Ligand Bound to FT Receptor	13	15	8.99e-02	7.97e-01
8	Hippo/Warts Pathway	15	18	9.31e-02	7.97e-01
9	Transport of Mature mRNAs (Intronless Transcripts)	33	46	1.04e-01	7.97e-01
10	Phosphorylation-Dependent Inhibition of YKI	7	7	1.07e-01	7.97e-01
11	Transport of Intronless Transcript mRNA	32	45	1.17e-01	7.97e-01
12	SLBP-Dependent Mature mRNA Transport	28	39	1.25e-01	7.97e-01
13	Eukaryotic Translation Elongation	6	6	1.29e-01	7.97e-01
14	SUMOylation of DNA Replication Proteins	27	38	1.41e-01	7.97e-01
15	SLBP-Independent Mature mRNA Transport	27	38	1.41e-01	7.97e-01
16	Chk1/Chk2-Mediated Inactivation of Cyclin B:Cdk1 Complex	8	9	1.43e-01	7.97e-01
17	Dephosphorylation of PER	8	9	1.43e-01	7.97e-01
18	SUMOylation of Chromatin Organisation Proteins	25	35	1.45e-01	7.97e-01
19	Transcriptional Regulation by Small RNAs	36	53	1.56e-01	7.97e-01
20	Prolonged ERK Activation Events	5	5	1.57e-01	7.97e-01

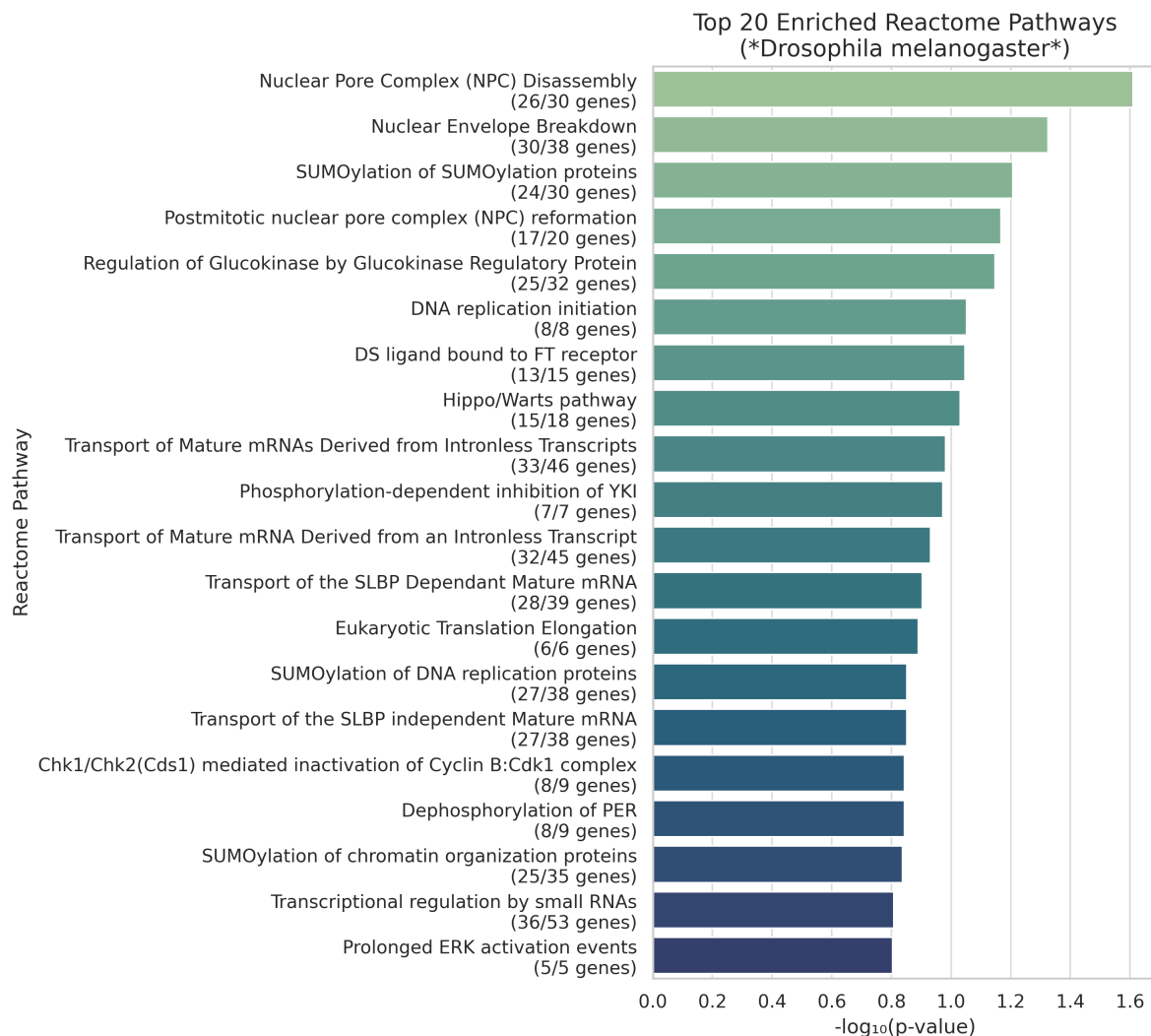


Figure 3.10 Visualisation of Top 20 enriched Reactome pathways

3.34. String protein-protein interaction and pathway network analysis

String functional enrichment analysis identified significant overrepresentation of developmental and regulatory biological processes within the mapped transcriptome-derived protein dataset (Figure 3.11). The most strongly enriched terms included pattern specification process, regionalisation, nervous system development, oogenesis and system development. Additional enriched processes included embryo development, cell fate commitment, cellular developmental processes and generation of neurons. The large number of genes associated with these categories likely reflects the whole-body nature of the larval transcriptome, which captures transcripts from multiple

tissues and developmental systems rather than focusing on a single organ or tissue. Collectively, these results indicate that the transcriptome contains a broad range of proteins involved in developmental regulation, tissue differentiation and cellular organisation.

Although developmental processes dominated the enrichment analysis, this was expected given the use of whole-body larval RNA. Consequently, enrichment results were further explored through pathway-level and protein-protein interaction analyses to identify conserved signalling modules and immune-associated functional networks within the dataset.

Following identification of enriched signalling pathways, overlap analysis was performed to determine which genes participated repeatedly across multiple enriched pathways. Genes occurring in numerous pathways may represent conserved signalling hubs involved in coordinating diverse biological processes.

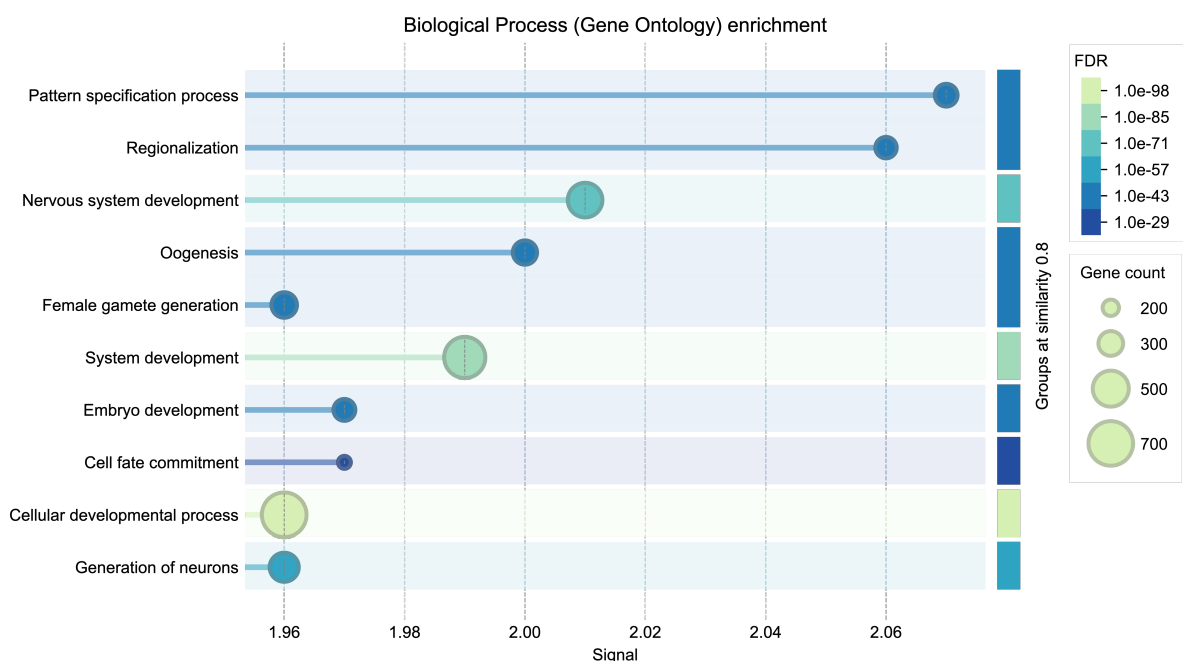


Figure 3.11 STRING functional enrichment visualisation of transcriptome-derived proteins mapped to *Drosophila melanogaster*. Bubble size represents the number of genes associated with each enriched biological process, while colour indicates the false discovery rate (FDR). Terms are ranked according to STRING signal strength.

3.35. Cross-pathway signalling hub analysis

To identify genes occupying central positions across enriched signalling pathways, genes identified within enriched KEGG, Reactome and WikiPathways datasets were ranked according to the number of curated pathway terms in which they occurred (Figure 3.12; Table 3.7). Ranking genes based on pathway recurrence enabled the identification of proteins repeatedly associated with multiple biological processes and highlighted candidates that may represent central hubs linking diverse signalling pathways.

The highest-ranked genes were predominately composed of ribosomal and proteasome-associated proteins. **RpL40** and **RpS27A** represented the most recurrent genes, occurring in 183 and 173 enriched pathway terms, respectively. Numerous proteasome subunits, including **Rpn6**, **Rpn7**, **Rpn2**, **Rpn8**, **Rpt2**, **Rpt6**, **Rpn10**, **Rpn11**, members of the **Prosalpha** family and **Prosbeta3** and **Prosbeta7**, were also repeatedly identified across more than 120 enriched pathways. The strong enrichment of these proteins reflects the widespread involvement of protein synthesis, ubiquitin-mediated protein degradation and proteostasis throughout diverse cellular pathways. These processes underpin virtually all aspects of cellular physiology and consequently recur across diverse signalling, metabolic and regulatory pathways represented within the annotated transcriptome.

Although structural and housekeeping proteins dominated the highest-ranking positions, several conserved signalling-associated proteins were also identified among the recurrent pathway genes (Table 3.7). These included **rl (Relish)** (70 pathway occurrences), **mts** (69), **Pp2A-29B** (68), **eff** (63), **Roc1a** (61), **Src64B** (60), **Akt1** (48) and **Rac1** (42). Unlike ribosomal and proteasome proteins, these genes function directly in intracellular signalling cascades and therefore represent more biologically informative candidates for signal integration across multiple pathways.

Relish (rl) is the terminal NF- κ B transcription factor of the Imd pathway and regulates antimicrobial peptide expression following Gram-negative bacterial recognition. **eff**

(effete/UbcD1) functions as a ubiquitin-conjugating enzyme required for ubiquitin-mediated protein regulation, an essential mechanism controlling signalling protein activation, degradation and immune pathway modulation. **Akt1** acts as a central kinase within the PI3K/Akt signalling pathway, coordinating cellular metabolism, growth, stress responses and survival. **Rac1**, a member of the Rho family of small GTPases, regulates cytoskeletal remodelling, phagocytosis, vesicular trafficking and multiple immune-related signalling processes. **Src64B** is a non-receptor tyrosine kinase implicated in signal transduction, cytoskeletal organisation and developmental signalling, while **mts** and **Pp2A-29B** encode catalytic and regulatory components of Protein Phosphatase 2A (PP2A), a conserved phosphatase complex responsible for modulating phosphorylation-dependent signalling across numerous cellular pathways. Collectively, the repeated identification of these signalling-associated proteins across multiple enriched pathways suggests that they function as important regulatory nodes integrating diverse biological processes.

Overall, this analysis demonstrates that genes recurring across numerous enriched pathways comprise both highly conserved housekeeping proteins and multifunctional signalling regulators. While ribosomal and proteasome components dominate the global pathway landscape because of their fundamental roles in protein homeostasis, recurrent signalling proteins such as **Relish**, **eff**, **Akt1**, **Rac1** and **Src64B** represent candidate hubs of signal transduction that link multiple biological pathways within the *Hermetia illucens* transcriptome. Collectively, these findings demonstrate that although housekeeping proteins dominate global pathway enrichment because of their essential cellular functions, conserved signalling regulators recur across numerous enriched pathways and therefore represent candidate signalling hubs capable of integrating multiple biological processes within the BSFL transcriptome.

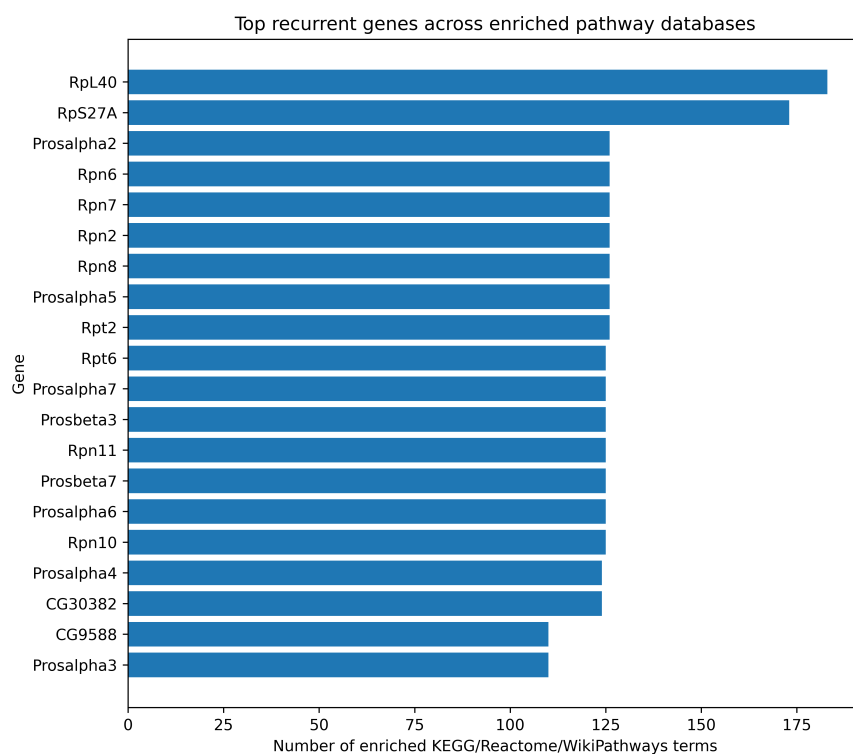


Figure 3.12 Top 20 recurrent genes identified across enriched KEGG, Reactome and WikiPathways databases. Genes are ranked according to the number of enriched curated pathway terms in which they occurred.

Table 3.7 Top 30 recurrent genes ranked according to the number of enriched KEGG, Reactome and WikiPathways terms in which they were identified.

Rank	Gene	Curated pathway occurrences
1	RpL40	183
2	RpS27A	173
3	Rpn6	126
4	Rpn7	126
5	Rpn2	126
6	Rpn8	126
7	Prosalpha5	126
8	Rpt2	126
9	Prosalpha2	126
10	Rpt6	125
11	Prosalpha7	125
12	Prosbeta3	125
13	Rpn11	125
14	Prosbeta7	125
15	Prosalpha6	125
16	Rpn10	125
17	Prosalpha4	124
18	CG30382	124
19	CG9588	110
20	Prosalpha3	110
21	PI31	109
22	CG12096	109
23	rl	70
24	mts	69
25	Pp2A-29B	68
26	eff	63
27	Roc1a	61
28	Src64B	60
29	Akt1	48
30	Rac1	42

3.36. Immune-associated protein interaction networks

To further investigate the biological significance of the annotated transcriptome-derived proteins, genes associated with immune-related enrichment categories were subjected to STRING protein-protein interaction (PPI) analysis. The resulting network consisted of 42 proteins connected by 157 predicted interactions (Figure 3.13A), substantially exceeding the four interactions expected by chance (PPI enrichment $p < 1 \times 10^{-16}$), indicating that the identified proteins are functionally connected and participate in shared biological processes rather than representing a random collection of annotated genes. Several proteins formed central hubs within the network. These included the pattern recognition receptors PGRP-LC, PGRP-LE, PGRP-SA, PGRP-LB, GGBP1 and GGBP3, which occupied prominent positions within the network and displayed extensive connectivity with downstream signalling proteins. These recognition molecules are known to function in the detection of microbial-associated molecular patterns and represent key components of insect innate immune responses.

Strong interactions were observed between multiple members of the canonical Imd signalling pathway. Central signalling proteins included imd, Rel, Dredd, Diap2, key (Kenny) and akirin. Particularly strong associations were identified between PGRP-LC and imd (combined score = 0.998), PGRP-LE and imd (0.997), Diap2 and imd (0.994), Dredd and imd (0.986), and Rel and imd (0.984), reflecting the conserved architecture of the Imd pathway described in *Drosophila melanogaster* and other insects. The presence of these interacting proteins suggests that major components of Gram-negative bacterial recognition and downstream NF- κ B signalling are represented within the transcriptome.

Several proteins associated with the Toll pathway were also identified. These included Toll-7, Tollo, spz4, grass, psh and modSP, which formed an interconnected cluster linked to recognition proteins and downstream immune regulators. The identification of both IMD-associated and Toll-associated components demonstrates the presence of multiple conserved innate immune signalling pathways within the transcriptome-

derived dataset. Downstream antimicrobial effectors were represented by Attacin-A (AttA), Attacin-C (AttC) and Diptericin-A (DptA). These proteins were highly connected to upstream recognition and signalling components, forming a distinct effector module within the network. Strong interactions were observed between DptA and AttC (combined score = 0.999), DptA and AttA (0.986), and AttA and AttC (0.976), indicating coordinated antimicrobial response mechanisms. Additional regulatory proteins, including LUBEL, Sarm, ECSIT, egr and akirin, were also present within the network and may contribute to immune regulation, signal integration and pathway crosstalk.

To further contextualise these interactions within established immune signalling cascades, proteins identified within the STRING network were examined alongside the canonical humoral immune response pathway for fungal and bacterial pathogens (Figure 3.13B). This pathway incorporates key components of the Toll, IMD and JAK/STAT signalling cascades and illustrates the progression from pathogen recognition through intracellular signal transduction to downstream antimicrobial effector responses. Components associated with pathogen recognition, including members of the PGRP and GGBP families, intracellular signalling proteins such as imd, Rel, Dredd and Diap2, and downstream antimicrobial effectors were represented throughout the pathway architecture, supporting the conservation of core innate immune mechanisms within the transcriptome-derived dataset. Collectively, these findings demonstrate that the transcriptome contains a substantial repertoire of conserved immune recognition, signalling and effector genes, supporting its utility as a resource for investigating innate immune pathways in *H. illucens*.

immune response pathway for fungal and bacterial pathogens in Drosophila melanogaster. The pathway integrates key components of the Toll, IMD and JAK/STAT signalling cascades involved in pathogen recognition, signal transduction and antimicrobial effector responses.

3.37. Cross-pathway signalling hub analysis: Immunity

To identify genes occupying central positions across enriched signalling pathways, STRING pathway analysis from KEGG, Reactome and WikiPathways was integrated and genes were ranked according to the number of enriched pathways in which they occurred (Table 3.8). Several immune-associated genes were repeatedly identified across multiple pathway categories, suggesting that they may function as hubs of signal transduction and pathway crosstalk.

The most frequently represented immune-associated genes included *eff* (63 pathways), *key* (40 pathways), *Rel* (39 pathways), *dorsal* (*dl*; 38 pathways), *Pelle* (20 pathways), *Hopscotch* (19 pathways) and *Dredd* (16 pathways). Many of these proteins occupy central positions within conserved innate immune signalling cascades. *Rel* and *Dorsal* are members of the NF- κ B family of transcription factors and act as major downstream regulators of antimicrobial and immune-responsive gene expression. Similarly, *Kenny* (*key*), *Dredd* and *imd* form core components of the IMD signalling pathway, while *Pelle*, *grass* and *psh* contribute to Toll pathway activation. The repeated occurrence of these genes across multiple enriched pathways highlights their importance as highly connected signalling intermediates.

Components of the JAK/STAT pathway, including *hop* and *dome*, were also identified among the recurrent pathway-associated genes, suggesting integration between immune signalling, developmental regulation and cellular homeostasis. Collectively, these findings indicate that a relatively small number of conserved signalling proteins are repeatedly utilised across multiple biological processes and pathway categories. Such genes are likely to represent key regulatory hubs within the BSFL transcriptome and may play important roles in coordinating immune responses, signal integration and downstream transcriptional regulation.

Overall, this analysis demonstrates that genes recurring across numerous enriched pathways comprise both highly conserved housekeeping proteins and multifunctional signalling regulators. While ribosomal and proteasome components dominate the global pathway landscape because of their fundamental roles in protein homeostasis, recurrent regulators such as Relish, eff, Akt1, Rac1 and Src64B represent candidate molecular hubs that integrate signalling, protein regulation and cellular homeostasis across multiple biological pathways within the *Hermetia illucens* transcriptome.

Table 3.8 Recurrence of canonical immune signalling genes across enriched KEGG, Reactome and WikiPathways terms. Genes are ranked according to the number of enriched pathway terms in which they occurred, highlighting conserved signalling regulators that participate in multiple immune-related pathways. Higher pathway occurrence indicates greater functional connectivity across the enriched signalling network.

Gene	Pathway occurrences
<i>eff</i> (<i>effete/UbcD1</i>)	63
<i>key</i> (<i>Kenny</i>)	40
<i>Rel</i>	39
<i>dl</i> (<i>Dorsal</i>)	38
<i>pll</i> (<i>Pelle</i>)	20
<i>hop</i> (<i>Hopscotch</i>)	19
<i>Dredd</i>	16
<i>imd</i>	6
<i>PGRP-LC</i>	6
<i>PGRP-LE</i>	5
<i>dome</i>	3
<i>PGRP-SA</i>	3
<i>psh</i>	3
<i>grass</i>	2

3.38. Discussion

This study presents a comprehensive genome-guided transcriptomic profile of the Black Soldier Fly (*Hermetia illucens*), with a specific focus on the identification and functional classification of genes related to innate immunity. Leveraging a high-quality chromosome-level reference genome and pooled whole-body Illumina RNA sequencing, 38,620 high-confidence transcripts were functionally annotated using BLASTP against the UniProt/Swiss-Prot database. From this, 191 immune-related genes were identified and categorised into canonical signalling pathways, notably Toll, IMD, and JAK/STAT, supporting the conservation of core immune architecture in *H. illucens*.

A large proportion of protein annotations were mapped to *Drosophila melanogaster* (15,394 hits), consistent with its role as the most experimentally validated model of insect innate immunity (Buchon, Silverman, & Cherry, 2014). While hits from mammals such as *Homo sapiens* and *Mus musculus* may initially appear unexpected, these likely reflect evolutionary conservation of fundamental cellular processes such as transcription regulation, signal transduction, and apoptosis, which are often mediated by highly conserved domains (Romero, Ruvinsky, & Yoac, 2014). Insect-relevant species, including *Aedes aegypti*, *Anopheles gambiae*, and *Bombyx mori*, were also represented, lending further weight to the biological relevance of annotated immune transcripts across invertebrates.

Immune-related transcripts were grouped into perception (n=37), integration (n=102), and response (n=46) categories based on functional annotation and keyword screening. While all major immune signalling pathways were represented, the disproportionate abundance of integration genes (e.g., MyD88, IMD, Hopscotch, Relish, STAT) likely reflects the highly conserved, modular nature of intracellular immune signalling components. These adaptor proteins, kinases, and transcription factors form the core signalling cascades that translate extracellular pathogen cues into cellular responses, such as antimicrobial peptide (AMP) production, inflammation, and apoptosis. Because these molecules are central to immune homeostasis and downstream activation, they are subjected to strong purifying selection, meaning their sequences change very little over time to preserve their function.

The relatively lower representation of pattern recognition receptors (PRRs) may result from lineage-specific receptor evolution or underannotation of BSF-specific variants in public databases. Pathogen-recognition receptors are typically under strong positive selection due to the evolutionary arms race between host immunity and microbial evasion strategies. Proteins such as effector molecules and recognition receptors must constantly adapt to detect a rapidly changing repertoire of pathogens. Consequently, this results in increased variability across species and lineages (Sackton, et al., 2007).

Notably, all key components of the Toll and IMD pathways were identified. These Toll pathway components were recovered with high-confidence BLASTP hits against Uniprot database, for example, Spätzle ($E = 2.88 \times 10^{-42}$; 48.6% identity), modSP ($E = 1.21 \times 10^{-74}$; 48.7%), Grass ($E = 6.63 \times 10^{-76}$; 55.4%), Toll ($E = 4.09 \times 10^{-140}$; 47.8%), and MyD88 ($E = 2.35 \times 10^{-105}$; 42.6%). Downstream effectors such as Pelle ($E = 1.23 \times 10^{-137}$; 55.0%) and the NF- κ B transcription factor Dorsal ($E = 4.94 \times 10^{-73}$; 37.2%) also showed strong matches, supporting deep evolutionary conservation of this canonical innate immune pathway in *Hermetia illucens*. This is also consistent with evidence from other dipterans such as *Aedes aegypti*, where activating the Toll pathway limits dengue virus (DENV) replication and dissemination, determined through RNAi-mediated silencing of inhibitors Cactus and Caspar and by showing that depletion of MyD88 increases the viral burden (Xi, Ramirez, & Dimopoulos, 2008). Similar outcomes have been shown when Toll is stimulated by Wolbachia-induced reactive oxygen species (ROS), further suppressing DENV in *Aedes aegypti* (Pan, et al., 2012). Functional genetics in *Aedes aegypti* show that toll signalling via REL1 is both sufficient to induce immune effectors (Spz1A, Serpin-27A) and necessary for antifungal resistance of *Beauveria bassiana*. REL1 knockdown increases susceptibility to *Beauveria bassiana*, establishing REL1 as the key downstream NF- κ B of the mosquito Toll pathway (Bian, Shin, Cheon, Kokoza, & Raikhel, 2005). In *Anopheles gambiae*, Toll signalling (Rel1) together with Rel2 establishes basal pre-invasion immunity by maintaining constitutive expression of complement-like effectors (TEP1, LRIM1). Concurrent knockdown of Rel1 and Rel2 suppresses TEP1/LRIM1 and increases *Plasmodium berghei* survival, whereas depletion of the NF- κ B inhibitor Cactus (thereby derepressing Rel1 by removing its cytoplasmic

inhibitor and allowing nuclear translocation) boosts TEP1/LRIM1 and completely aborts parasite development (Frolet, Thoma, Blandin, Hoffmann, & Levashina, 2006).

In parallel, we recovered the core IMD cascade that senses DAP-type peptidoglycan from Gram-negative bacteria via membrane-bound PRRs such as PGRP-LC or PGRP-LE, driving antibacterial peptide expression. This includes oligomerisation, recruitment of IMD, interaction with FADD, and DREDD-mediated cleavage of Relish, which translocate to the nucleus and activate AMP genes including Attacins, Diptericins, and Cecropins (Stoven, et al., 2003). Candidate hits include the cytosolic/membrane pathogen recognition receptor (PRR) PGRP-LE ($E = 1.86 \times 10^{-78}$; 45.1% identity), the central adaptor IMD (5.06×10^{-36} ; 43.5%), FADD (1.46×10^{-29} ; 32.0%), the initiator caspase DREDD (7.54×10^{-90} ; 37.8%), and the NF- κ B transcription factor Relish/Rel (1.13×10^{-122} ; 38.2%). Together, these suggest a conserved caspase-dependent NF- κ B module in *H. illucens*, consistent with the *Drosophila* pathway in which DREDD cleaves Relish to induce IMD-responsive AMPs (e.g., Diptericins/Attacins) (Stoven, et al., 2003). Mosquito work using *Anopheles stephensi* showed how IMD/Rel2 directed an anti-Plasmodium and antibacterial programme (Dong, Das, Cirimotich, McLean, & Dimopoulos, 2011).

Further supporting the immune competence of *H. illucens*, we identified the full JAK/STAT pathway architecture, including the receptor Domeless (Dome) ($E = 5.52e-103$; 38.5% identity), the Janus kinase Hopscotch (Hop) ($E = 2.39e-104$; 39.8%), the transcription factor Stat92E ($E = 2.05e-133$; 47.4%), and the negative regulator SOCS36E ($E = 2.55e-58$; 47.9%). This Dome–Hop–STAT–SOCS configuration mirrors the canonical dipteran paradigm, where Upd-like cytokines stimulate immune and stress-responsive gene expression in haemocytes, gut, and fat body (Yu, Luo, Xu, Zhang, & Jin, 2022). In *Drosophila melanogaster*, JAK/STAT signalling plays an essential role in antiviral immunity and haemocyte proliferation, with Upd2 and Upd3 acting as damage signals during infection or tissue stress. Conservation of these core members in *H. illucens* suggests potential for similar immunoregulatory roles, although further functional work is needed to define ligands and target genes. In *Aedes aegypti*, JAK/STAT activation

contributes to antiviral defence against Dengue virus (DENV), and its inhibition enhances viral replication (Souza-Neto, Sim, & Dimopoulos, 2009)

While functional validation is needed, the transcriptomic evidence supports the existence of canonical Toll, IMD and JAK/STAT signalling modules in BSF, potentially governing responses to bacterial, fungal and parasitic challenges.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses provided functional context for the transcriptomic dataset, highlighting overrepresented molecular processes and immune-relevant pathways within the filtered transcriptome of *Hermetia illucens*. Go enrichment revealed significant enrichment across all three ontology domains: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC), offering insight into the cellular functions, molecular activities, and localisations of the expressed genes.

Across Biological Process terms, several annotations were consistent with immune activation, including defence response, response to bacterium, and regulation of transcription in response to stress. These enrichments reflect the broad transcriptional activity of larvae exposed to complex rearing substrates, many of which contain microbial consortia capable of stimulating host immunity. Enrichment of terms related to innate immune response, signal transduction, and cell communication further support the inference that immune-relevant genes were captured in this transcriptomic profile.

In the Molecular Function category, terms such as ATP binding, protein kinase activity, and serine-type endopeptidase activity were prominent. These are often associated with signal integration machinery such as kinases, proteases, and transcription factors, corresponding with earlier observations that integration genes are overrepresented in the immune gene list.

Cellular Component enrichment identified terms such as membrane, cytoplasm, and nucleus, consistent with the known subcellular locations of immune signalling events. Membrane-associated annotations likely represent pattern recognition receptors

(PRRs), while nuclear terms reflect the transcriptional output driven by NF- κ B or STAT family transcription factors.

KEGG pathway enrichment largely overlapped with GO findings, with overrepresented pathways including MAPK signalling, apoptosis, lysosome function, and protein processing in the endoplasmic reticulum, all relevant to immunity, cellular defence, and stress response. Terms such as lysosome and phagosome are particularly interesting, as they support cellular immune functions such as microbial engulfment and degradation.

Together, the GO and KEGG results validate the core annotations derived from BLASTP filtering and keyword classification. While functional enrichment did not always pass FDR correction due to multiple testing and the non-model status of *H. illucens*, the strong overlap with known immune and stress-associated pathways builds confidence in the biological relevance of the transcriptome. Furthermore, *Drosophila melanogaster* was used as the functional reference background during g:Profiler enrichment analysis, enabling improved mapping to GO and KEGG pathways due to its well-annotated immune system and shared dipteran lineage.

Pathway enrichment analysis via g:Profiler and Reactome, using FlyBase-mapped orthologs, further confirmed the overrepresentation of pathways linked to immune signalling, mRNA transport, SUMOylation, and stress responses, although no pathway passed FDR correction. This may reflect the broad functional scope of the input gene set and underscores the challenge of assigning pathway-level significance in non-model organisms.

Interestingly, several of the immune transcripts exhibited high E-values and/or low percent identity (<30%), suggesting the possibility of divergent or truncated homologs. While these retained strong E-values overall, they should be considered lower-confidence annotations and potential candidates for experimental validation, particularly if they encode proteins with unusual structure or limited domain conservation.

The curated immune gene list provides a foundation for future research aiming to monitor BSF health, explore immune activation under challenge conditions, and select for disease-resistant strains. Given the increasing use of BSF in waste bioconversion, animal feed, and antimicrobial peptide discovery, a better understanding of its immune system is critical. This is particularly relevant as industrial rearing conditions may increase susceptibility to microbial imbalance or infection, necessitating tools for early detection and intervention.

Beyond pathway enrichment alone, STRING protein–protein interaction analysis demonstrated that many of the annotated proteins formed highly interconnected interaction networks rather than existing as isolated components. The highly significant enrichment of the protein-protein interaction network supports the biological validity of the annotation strategy and indicates that the identified orthologues form coherent functional modules rather than representing isolated gene annotations. These findings strengthen confidence that the transcriptome captures integrated biological systems rather than collections of independently annotated genes.

Cross-pathway hub analysis further demonstrated that a relatively small number of genes were repeatedly represented across numerous enriched KEGG, Reactome and WikiPathways terms. Although housekeeping proteins associated with the ribosome and proteasome dominated the highest-ranking positions owing to their fundamental roles in protein synthesis and proteostasis, several multifunctional regulators including Relish, eff, Akt1, Rac1 and Src64B were consistently recovered across diverse biological pathways. This predominance is expected because ribosomal proteins and proteasome components participate in numerous fundamental cellular processes and consequently recur across diverse enriched pathways irrespective of the specific biological process being examined. In contrast, the repeated identification of signalling-associated proteins suggests that these genes occupy central regulatory positions capable of integrating immune signalling with broader cellular processes. Such genes therefore represent attractive candidates for future functional validation because modulation of these regulatory hubs would be expected to influence multiple interconnected biological pathways simultaneously. While this study focused on

transcript-level evidence, future work should aim to integrate proteomics, single-cell RNA-seq, or CRISPR-based validation to confirm the expression, function, and tissue-specific activity of candidate immune genes. Comparative studies across different BSF strains, developmental stages, and microbial exposures would also provide valuable insights into immune plasticity and strain-specific responses.

In conclusion, this study expands the known repertoire of immune-relevant genes in *Hermetia illucens*, reinforces the conservation of core insect immune pathways, and demonstrates that integrating genome-guided annotation with functional enrichment, protein-protein interaction analysis and cross-pathway hub identification provides a systems-level framework for characterising innate immunity in this emerging industrial insect. These findings offer a valuable transcriptomic resource for the BSF research community and have direct implications for BSF health monitoring, strain improvement and the development of immune-informed breeding and biosecurity strategies.

Chapter 4. mRNA abundance of antimicrobial peptides (AMPs) and pattern-recognition receptors (PRRs) in immune-challenged *Hermetia illucens* larvae following lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (Poly (I:C)) exposure

4.1. Antimicrobial Peptides

Antimicrobial Peptides (AMPs) are evolutionarily conserved, short-chain polypeptides that form an essential component of the innate immune system across nearly all domains of life, including plants, animals and microorganisms (Bin Hafeez, Jiang, Bergen, & Zhu, 2021). Unlike traditional antibiotics, AMPs possess broad-spectrum activity against bacteria, fungi, parasites, and some viruses, functioning through rapid membrane disruption or interference with intracellular targets (Mahlpuu, Håkansson, Ringstad, & Björn, 2016). Their ability to modulate immune responses, control inflammation, and promote wound healing has made them the subject of growing interest in both basic immunology and applied clinical research (Cresti, Cappello, & Pini, 2024).

The rise of antimicrobial resistance (AMR) in pathogenic microorganisms has created an urgent need for alternative therapeutic strategies, positioning AMPs as potential next-generation antibiotics (Salam, et al., 2023) (Xuan, et al., 2023). Several AMPs are now undergoing clinical evaluation for their efficacy in treating drug-resistant infections, chronic wounds, and inflammatory diseases (Magana, et al., 2020). Despite challenges associated with peptide stability, delivery, and production cost, the structural diversity and functional versatility of AMPs continues to fuel significant biomedical and biotechnological interest.

Insects have emerged as a rich and largely untapped source of AMPs, owing to their diverse habitats and co-evolution with microbial communities (Dho, Candian, & Tedeschi, 2023). Saprophagous species such as the Black Soldier Fly (*Hermetia illucens*) are frequently exposed to high microbial loads due to their role in decomposing organic waste, necessitating a highly efficient innate immune system (Vogel H. , Müller, Heckel, Gutzeit, & Vilcinskis, 2018) (Van Moll, et al., 2022). The transcriptome of *H. illucens* has revealed a surprisingly high number of genes encoding AMPs, including defensins, cecropins and attacins, some of which are functionally unique among insects (Vogel H. , Müller, Heckel, Gutzeit, & Vilcinskis, 2018) (Fahmy, 2023) (Liu, et al., 2024).

As interest in insect-based bioconversion systems continues to rise, understanding the molecular underpinnings of immunity in these species, and how AMPs are regulated and expressed under immune challenge, is critical for improving production systems and unlocking new applications.

This chapter will explore the current research, functional diversity, and regulatory landscape of AMPs, with particular emphasis on their role in insects and the Black Soldier Fly. It will also highlight recent advances in AMP characterisation, including experimental approaches to studying immune AMP mRNA abundance using molecular mimics: Pathogen-Associated Molecular Patterns (PAMPs) like lipopolysaccharide (LPS) and Poly (I:C).

4.2. Current Uses of Antimicrobial Peptides (AMPs) in Research and Clinical Practice

In human medicine, topical formulations of AMPs have shown effectiveness in treating chronic wounds, such as diabetic foot ulcers, pressure sores, and burn injuries, due to their antimicrobial activity and ability to accelerate tissue repair (Lipsky, Holroyd, Zasloff, & Pollak, 2008). Several synthetic AMPs, including pexiganan (a magainin analogue) and omiganan (a derivative of indolicidin), have reached advanced clinical trial stages for applications. Pexiganan has undergone Phase III clinical trials for treating diabetic foot ulcers, where it demonstrated broad-spectrum antimicrobial activity and enhanced wound healing without contributing to resistance development (Lipsky, Holroyd, Zasloff, & Pollak, 2008). Similarly, omiganan, derived from bovine indolicidin, has been evaluated for catheter-associated infections and acne, showing strong efficacy against *Staphylococcus* spp. and *Cutibacterium acnes* while maintaining low cytotoxicity (Magana, et al., 2020) (Yu, et al., 2017). Beyond infectious disease control, AMPs are under investigation for their immunomodulatory properties. Some peptides can act as signalling molecules that modulate inflammation, promote angiogenesis, or recruit immune cells to sites of infection, suggesting utility in inflammatory and autoimmune disorders (Mookherjee, Anderson, Haagsman, & Davidson, 2020).

In veterinary and animal production settings, AMPs are increasingly explored as alternatives to prophylactic antibiotics. They have demonstrated promising results in reducing pathogen load and improving gut health in livestock, poultry, and aquaculture species, key to reducing reliance on antibiotic growth promoters (Choi, Silva, Song, & Lee, 2013) (Rajanbabu & Chen, 2011). For example, supplementation with insect-derived AMPs has been linked to improved resistance to *Vibrio* spp. and *Aeromonas hydrophilia* in fish, and the reduced colonisation of pathogenic *Escherichia coli* in poultry (Li, et al., 2021). In aquaculture, where AMR poses a significant threat to food security, insect-derived AMPs have shown promise as functional feed additives and immune stimulants. The antimicrobial peptide epinecidin-1, a cationic AMP originally isolated from the orange-spotted grouper (*Epinephelus coioides*) significantly increased survival, lysozyme activity, and bacterial clearance following *Vibrio vulnificus* infection in mice (Chuang, Chen, Lin, & Chang, 2011). Similarly, insect-derived peptides have been successfully applied to protect carp and tilapia from *Aeromonas hydrophilia*, highlighting their immunostimulatory as well as antimicrobial properties (Choi, Silva, Song, & Lee, 2013). In the context of animal agriculture, AMPs are increasingly explored as antibiotic alternatives. For example, supplementation of chicken feed with recombinant defensins in poultry feed led to a reduction in *Escherichia coli* colonisation and improved intestinal morphology (Li, et al., 2021). These results underline AMPs' dual role in pathogen suppression and host health promotion, providing a sustainable alternative to prophylactic antibiotic use.

The utility of AMPs is also expanding into biomaterials and surface engineering. AMP coatings are being applied to catheters, surgical meshes, and wound dressings to reduce infection risks in hospital settings, a growing concern with the rise of hospital-acquired infections (Costa, Gomes, Reis, & Martins, 2019). Advances in nanotechnology and peptide engineering are also addressing historical limitations of AMPs, including their susceptibility to protease degradation, cytotoxicity at high doses, and challenges in large-scale production (Fjell, Hiss, Hancock, & Schneider, 2012).

Collectively, these case studies support the growing body of evidence that AMPs offer robust, multi-functional solutions across diverse biological systems. However,

scalability, cost-effective synthesis, and regulatory approval remain limiting factors, necessitating continued research and refinement of AMP production platforms, including those derived from sustainable insect sources like *Hermetia illucens*.

4.3. Legislative and Regulatory Considerations Surround AMPs

Despite their promising potential, the development and commercialisation of antimicrobial peptides (AMPs) face several regulatory and legislative hurdles. These challenges stem from AMPs' unique nature as biologically active peptides, which places them in a regulatory grey area between conventional pharmaceuticals, biologics and feed additives, depending on their intended application.

In pharmaceutical development, AMPs are typically classified as biological medicinal products, which means they must meet strict safety, efficacy, and quality requirements before approval. In the European Union, this falls under Directive 2001/83/EC and Regulation (EC) No 726/2004, which govern medicines for human use. The U.S Food and Drug Administration (FDA) similarly evaluates AMPs under the Investigational New Drug (IND) framework, often requiring extensive preclinical and clinical testing. These processes can be lengthy and costly, particularly for AMPs with novel mechanisms or non-traditional delivery systems (Mahlapuu, Håkansson, Ringstad, & Björn, 2016).

When AMPs are used in animal feed or agriculture, their classification can vary. Insects such as *Hermetia illucens* are now being explored not only as feed ingredients but also for their immunomodulatory bioactives, including AMPs. However, legislation is often ambiguous. For instance, the European Food Safety Authority (EFSA) currently regulates insect-derived feed under tight constraints, focusing primarily on protein and fat composition. AMP content is not yet a formal consideration, despite emerging research suggesting benefits in disease resistance and gut health (EFSA Panel on Nutrition, Novel Foods and Food Allergens, 2021).

In food applications, the inclusion of AMPs as preservatives or antimicrobial agents presents further legal complexity. While several bacteriocins (e.g., nisin) are approved

as food additives, insect-derived AMPs are not currently recognised in any major regulatory jurisdiction for direct food use. Safety concerns around allergenicity, stability, and unintended microbiome impacts have slowed progress (Singh, et al., 2023).

As previously mentioned, the use of AMPs in medical devices and surface coatings, particularly in hospitals, has potential for preventing microbial colonisation (Kazemzadeh-Narbat, et al., 2021). Biocompatibility of a novel AMP coating technology can be referenced against the ISO 10993 series; however, these uses often require separate regulatory evaluations as medical devices or combination products, which vary across countries and regions.

In summary, while the therapeutic and industrial potential of AMPs is clear, inconsistent and evolving regulations present a major challenge to their integration into mainstream markets. Moving forward, clearer regulatory frameworks that accommodate both synthetic and naturally derived AMPs, particularly from novel sources like insects, will be essential for unlocking their full value.

4.4. Antimicrobial Peptides (AMPs) in Insects

Insects rely primarily on innate immunity to defend against pathogens with antimicrobial peptides (AMPs) forming a critical part of their humoral immune response. Unlike vertebrates, insects lack adaptive immunity and instead mount rapid, non-specific defences, including the production of AMPs upon pathogen exposure. These peptides are typically synthesised in the fat body, the insect equivalent to the vertebrate liver, which is then secreted into the haemolymph following immune stimulation via pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), β -glucans, and peptidoglycan fragments (Bulet, Hetru, Dimarcq, & Hoffmann, 1999).

AMPs in insects are structurally diverse but can be broadly classified into major families based on their biochemical properties and mechanisms of action. These include:

- **Defensins** – cysteine-rich peptides that disrupt bacterial membranes particularly effective against Gram-positive bacteria.
- **Cecropins** - α -helical peptides with broad-spectrum activity, often upregulated following Gram-negative bacterial infection.
- **Attacins and Dipterocins** - glycine-rich peptides involved in targeting Gram-negative bacteria.
- **Drosomycins and Metchnikowins** - peptides with strong antifungal activity, particularly in species such as *Drosophila melanogaster* (Hoffmann & Reichhart, 2002).

These AMPs are regulated primarily through two canonical immune pathways: the Toll pathway and the IMD (Immune Deficiency) pathway. The Toll pathway is typically activated by Gram-positive bacteria and fungi, leading to the expression of defensins and drosomycins, while the IMD pathway responds to Gram-negative bacteria, triggering the expression of cecropins, dipterocins, and attacins (Lemaitre B. &, 2007). Crosstalk and redundancy between these pathways also allow insects to mount effective responses to mixed infections.

AMP expression can be both systemic and localised. For example, local expression in the gut or tracheae helps maintain epithelial barrier integrity and regulate symbiotic microbial communities (Tzou, et al., 2000). Moreover, recent research has revealed that certain insect AMPs also exhibit immunomodulatory roles, such as recruiting haemocytes (insects' leukocyte equivalents), modulating inflammation, and participating in tissue repair (Mylonakis, Podsiadlowski, Muhammed, & Vilcinskis, 2016).

The study of AMPs in insects has been pivotal to understanding innate immunity more broadly. The 1995 Nobel Prize-winning discovery of the Toll pathway in *Drosophila* laid the foundation for uncovering similar signalling mechanisms, TLR pathways, in mammals. Thus, insects continue to serve as indispensable models for AMP discovery and, in the wider sense, comparative immunology.

4.5. Using LPS (lipopolysaccharide) and Poly(I:C) (polyinosinic:polycytidylic acid) to map PRR-driven changes in antimicrobial peptides (AMPs) and pathogen recognition receptors (PRRs) mRNA abundance in BSFL

The use of LPS (lipopolysaccharide) and Poly(I:C) (polyinosinic:polycytidylic acid) as immune stimulants in *Hermetia illucens* (Black Soldier Fly Larvae, BSFL) was implemented to test mRNA abundance, particularly in antimicrobial peptides (AMPs) and pathogen recognition receptors (PRRs). This approach offers several advantages over traditional bacterial cultures, including increased experimental control, faster immune activation, and the ability to target specific pathways.

LPS is a major component of the outer membrane of Gram-negative bacteria and serves as a potent pathogen-associated molecular pattern (PAMP) (Farhana & Khan, 2023). It is recognised by the immune system through Toll-like receptor 4 (TLR4) in vertebrates (although not present in fish (Patli, 2011)); however, in insects like BSFL, LPS recognition is mediated through different pathways, including peptidoglycan recognition proteins (PGRPs) and other pattern recognition receptors (PRRs). Upon detection, LPS triggers immune signalling cascades such as the Toll and IMD (Immune deficiency) pathways, leading to the activation of antimicrobial peptide genes like Defensins, Cecropins, and Attacins. LPS stimulation is particularly useful in BSFL because it provides a rapid and standardised method to mimic bacterial infection without requiring live pathogens.

Poly (I:C) is a synthetic analogue of double-stranded RNA (dsRNA), which is commonly associated with viral infections. It is recognised by Toll-like receptor 3 (TLR3) in vertebrates, but in insects, it is detected by RNA interference (RNAi) pathways and possibly Dicer-2, an enzyme responsible for dsRNA cleavage. Poly(I:C) leads to the induction of antiviral responses, including the production of RNAi-related effectors and immune signalling molecules.

Using Poly(I:C) serves as an important tool to study the immune response against viral-like infections (Stowell, et al., 2009). Unlike live viral infections, which can be difficult to control and may vary in replication efficiency, Poly(I:C) provides a consistent and quantifiable method to examine how larvae respond to ds-RNA-related immune triggers. This is particularly useful in studying genes involved in innate antiviral immunity, stress responses, and crosstalk between bacterial and viral defence mechanisms.

Understanding how BSFL respond to LPS and Poly(I:C) contributes not only to basic insect immunology, but also to applied research in bioconversion, waste management, and alternative protein production. BSFL are increasingly being explored as a sustainable feed source, and their immune responses play a crucial role in determining their resilience to microbial challenges. By characterising immune gene expression under different conditions, studies can continue to explore various strategies to enhance the survival and efficiency of BSFL production systems, including gaining insights into host-pathogen interactions, innate immune evolution, and potential applications in biocontrol strategies against insect-borne diseases.

Candidate genes selected for transcriptional analysis were informed by the immune gene resource generated in Chapter 3, which identified candidate genes involved in pathogen perception, signal integration, and immune response. Defensins, attacins, and cecropins were selected because they represent major AMP families previously identified in *H. illucens* and are known to play important roles in antibacterial defence in insects. TLR6 was selected as a candidate pathogen recognition receptor due to its putative role in immune sensing and signal initiation, allowing investigation of potential transcriptional changes occurring at the recognition stage of the immune response. Together, these targets allowed investigation of both upstream pathogen recognition events and downstream antimicrobial effector responses following immune stimulation.

Building upon the immune gene resource developed in Chapter 3, this chapter aimed to determine whether bacterial and viral PAMP stimulation induces measurable

transcriptional changes in selected pathogen recognition receptors and antimicrobial peptides in *H. illucens*. By examining genes involved in both pathogen recognition and downstream effector responses, this work sought to identify molecular markers of immune activation and improve understanding of innate immune regulation in BSFL.

4.6. Materials and Methods

4.7. Housing and Handling of *Hermetia illucens*

Black soldier fly larvae (*Hermetia illucens*, BSFL) were shipped overnight in vented containers with feed (barley, rolled oats, water) and heat packs. On arrival, larvae were kept in the dark at 25°C. Ethics approval under AWERB 2023 12442 11402.

4.8. Immune stimuli (PAMPs) and experimental groups

Lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid [Poly(I:C)] were used to mimic bacterial and viral challenge, respectively. Dose series for each PAMP were 2 µg, 200 ng, 20 ng, and 2 ng per larva (in phosphate-buffered saline, PBS). Control conditions were untreated (no handling), sham injection (needle only; “IO”), and vehicle (PBS, 10 µL). n=10 per treatment, all of which were repeated in triplicate. Furthermore, each replicate was repeated with a new generation of larvae to account for genetic variability.

4.9. Microinjection procedure and Sample Capture

Larvae were placed on a chilled surface (ice) for brief immobilisation. A sterile, fine-gauge micro syringe/needle was used to deliver 10 µL into the hemocoel at the penultimate metamere. After treatment, larvae grouped according to their treatment plan into Petri dishes, sealed with parafilm, and maintained at 25 °C. At the 24 h point, larvae were flash frozen in liquid nitrogen and stored at -80°C for downstream analysis.

4.10. RNA extraction and cDNA synthesis

Whole larvae (frozen) were left to defrost on ice and dissected into three segments longitudinally following the Amended TRIzol protocol described in Chapter 2. RNA purity (A260/280), integrity (agarose gel and Bioanalyzer), and yield were recorded. cDNA was synthesised with the QIAGEN QuantiTect Reverse Transcription Kit. Using 1 µg of template RNA with 2 µL of gDNA Wipeout Buffer, 7x and RNase-free water (variable) to make up to a total of 14 µL. After which, the inclusion of 1 µL of Quantiscript Reverse Transcriptase, 4 µL Quantiscript RT Buffer, 5x, 1 µL RT primer mix and the entire 14 µL from the previous gDNA elimination reaction allowed for complete reverse transcriptase of all samples. These were validated through PCR using *Hermetia illucens* housekeeping gene RPL5 (60S ribosomal protein L5).

4.11. Target Selection, Sequence Screening and Primer Design

Seven genes were taken forward for qPCR after in-silico and bench screening: three defensins (Defensin-A-like, Defensin-B-like, Defensin-like peptide-1), one cecropin (CLP1), two attacins-A-like paralogues, and Toll-like Receptor 6 (TLR6). Sequences were collated from (Vogel H. , Müller, Heckel, Gutzeit, & Vilcinskas, 2018) and (Candian, Meneguz, & Tedeschi, 2023) additional candidates were dropped if specificity/efficiency were suboptimal.

In silico screening included full ORF (Open Reading Frames) using ExPASy Translate, Antimicrobial Peptide Scanner VR.2, SignalP-5.0 to predict Sec/SPI signal peptides with cleavage sites and structural analysis using AlphaFold. In the case of TLR6, structural analysis was determined through InterProScan which consolidates multiple signatures across databases (PFAM/SMART/PROSITE), and TMHMM/Phobius.

4.12. Sequence Alignment

Sequence Alignment was performed for Defensins and Attacins using Clustal Omega (visuals through ESPript 3.0) and pairwise alignment through NCBI BLAST for detailed insights into sequence similarities, identities, and conserved regions.

4.13. Primer Optimisation

All primers were validated in silico, and thermal gradient PCR was performed to optimise annealing temperatures to ensure no nonspecific amplification or abnormal results. The *Hermetia illucens* housekeeping gene RPL5 (60S ribosomal protein L5) was used as a reference to validate primer performance.

4.14. Molecular Cloning

4.15. PCR Product Purification

PCR products were purified to prepare DNA for cloning. Samples were adjusted to 50 µL with Milli-Q water, and 100 µL of NTI buffer was added for DNA binding. The mixture was transferred to PCR clean-up columns and centrifuged at $11,000 \times g$ for 30 seconds. After discarding the flow-through, the columns were washed with 700 µL NT3 buffer, centrifuged, and dried by further centrifugation. DNA was eluted with 15 µL of pre-heated NE buffer and quantified using a NanoDrop spectrophotometer.

4.16. Cloning into pGEM-T Easy Vector

Ligation reactions were set up with 5 μ L of 2 $\times$ Rapid Ligation Buffer, 1 μ L of pGEM-T Easy vector (50 ng), 3 μ L of PCR product, and 1 μ L of T4 DNA ligase in a total volume of 10 μ L. The reactions were incubated overnight at 4 °C.

4.17. Transformation into XL-1 Blue Competent Cells

Competent cells were thawed on ice, and 50 μ L aliquots were mixed with 0.85 μ L of β -mercaptoethanol. After a 10-minute incubation on ice, 5 μ L of the ligation reaction was added to the cells. The mixture was incubated on ice for 30 minutes, heat-shocked at 42 °C for 45 seconds and placed back on ice for 2 minutes. LB medium (300 μ L, without antibiotic) was added, and the cells were incubated at 37 °C for 1 hour in a shaker.

Transformed cells (150 μ L) were plated on LB agar containing ampicillin, IPTG, and X-gal, and the plates were incubated overnight at 37 °C.

4.18. Colony Screening and Plasmid DNA Extraction

White colonies were picked and cultured in 5 mL of LB medium with ampicillin. After overnight incubation at 37 °C, glycerol stocks were prepared by mixing 100 μ L of bacterial culture with 100 μ L of glycerol-LB medium and stored at -20 °C.

Plasmid DNA was extracted using a spin-column method. Bacterial cultures were centrifuged, and the pellets were lysed with buffers A1, A2, and A3. The lysate was cleared by centrifugation and loaded onto spin columns. After washing and drying the columns, DNA was eluted with 50 μ L of pre-heated AE buffer and quantified using a Nanodrop spectrophotometer.

4.19. Verification and Sequencing

Extracted plasmid DNA was verified by PCR, and samples were prepared for sequencing by combining 5 µL of plasmid DNA with 5 µL of T7 primer. Labelled tubes were submitted for sequencing analysis.

4.20. qPCR analysis

qPCR was performed on a qTOWER real-time PCR system (Analytik Jena) using SYBR Green chemistry. For each reaction, a master mix (18 µL per well) was prepared as follows: 10 µL 2x SYBR master mix, 7 µL nuclease-free water, 0.5 µL forward primer, and 0.5 µL reverse primer (final master-mix volume 18µL). To each well, 2 µL of template (cDNA sample, standard (10^7 - 10^1), or nuclease-free water for NTC) was added to give a final reaction volume of 20 µL. All samples were run in triplicate.

Reactions were run with the instrument's default SYBR settings and a target-specific annealing/extension temperature (as optimised for each primer pair), followed by a melt-curve to verify single, specific products. For absolute quantification, Ct values for standards were regressed against $\log_{10}(\text{copy number})$, and unknowns were interpolated from the resulting standard curve. Curves were accepted when efficiency was 90–110% and $R^2 \geq 0.99$.

qPCR data were also analysed using absolute copy number and fold-change estimates. Absolute copy number was used for visualisation of individual biological replicates across treatment and dose groups. Fold-change values were calculated for LPS and Poly(I:C) treatments relative to both untreated control and PBS-injected conditions at each dose. Dose-response relationships were assessed using Spearman correlation and linear regression. Treatment effects were further assessed using linear models including dose as a covariate, with additional treatment x dose interaction models used to determine whether immune stimulation responses varied across dose.

4.21. qPCR data visualisation

All analyses were performed in R (4.4.x) using tidyverse, ggplot2, Rmisc, car, ggsci, gridExtra, agricolae, ggpubr, and rstatix. Raw per-well exports (CSV) containing copy numbers, treatment group, and concentration were imported and organised into a tidy format. To stabilise variance, copy numbers were log-transformed (log10). Factor levels were explicitly ordered for Treatment (Control, PBS, POLY (I:C), LPS) and Concentration (2 µg, 200 ng, 20 ng, 2 ng), with optional injection-only runs using IO subsets.

Normality of raw and transformed data was assessed by Q–Q plots and Shapiro–Wilk tests. Because assumptions were not met, we used Kruskal–Wallis tests on log10(copy number) followed by pairwise Wilcoxon tests (Benjamini–Hochberg adjustment) implemented via rstatix; only adjusted $p < 0.05$ contrasts were annotated. Visualisation used ggpubr::ggboxplot (box + jitter), and significance brackets added via ggpubr::stat_pvalue_manual using y-positions computed by rstatix::add_xy_position. Figures were exported as high-resolution PNGs (300–600 dpi) with fixed dimensions for reproducibility.

4.22. Results

To investigate the immune response of *Hermetia illucens* larvae to bacterial and viral mimics, a panel of candidate antimicrobial peptides (AMPs) were selected for qPCR screening to quantify mRNA abundance across different concentrations of lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid [Poly(I:C)] within a defined timeframe.

A stepwise workflow was implemented to select and validate suitable targets: transcript sequences were extracted (Figure 4.1) (Vogel H. , Müller, Heckel, Gutzeit, & Vilcinskis, 2018) (Candian, Meneguz, & Tedeschi, 2023), pairwise alignments were conducted to distinguish paralogues, AMP candidacy was confirmed through in silico prediction tools, primers were designed and optimised, and thermal cycling conditions were

refined prior to qPCR screening. The following sections describe each of these stages in detail.

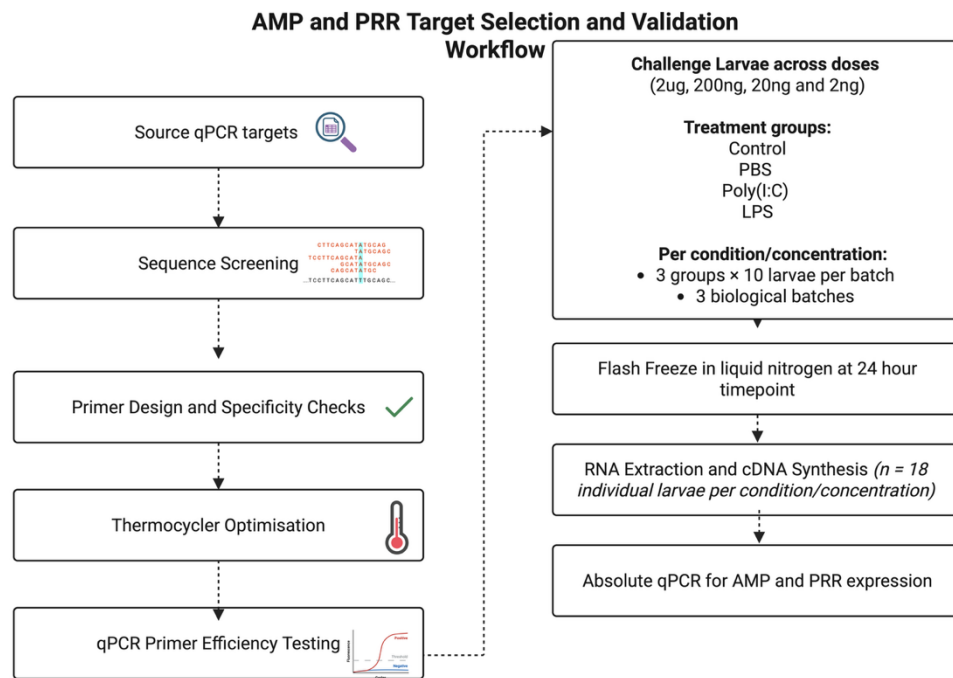


Figure 4.1 Workflow illustrating the selection, validation, and qPCR screening of antimicrobial peptide (AMP) and pathogen recognition receptor (PRR) targets in *Hermetia illucens*.

4.23. Defensin AMP Selected for Transcript Screening

Among the antimicrobial peptide (AMP) candidates selected for transcript-level investigation were Defensins. These candidates were chosen based on prior annotation within the *Hermetia illucens* transcriptome assembled by Vogel and colleagues (Vogel H. , Müller, Heckel, Gutzeit, & Vilcinskis, 2018) and an additional independently published defensin sequence described by (Candian, Meneguz, & Tedeschi, 2023).

Each defensin represents a distinct subtype: Defensin-A-like isoform (NCBI Reference Sequence: XM_038070404.1, [LOC119661185]), Defensin-B-like isoform (HERIL_Hill_BB_C34351), and defensin-like peptide 1 (HERIL_Hill_BB_C7176).

These sequences were selected to explore mRNA abundance following immune stimulation with bacterial and viral mimics. To mitigate the risk of cross-amplification in

downstream assays, all three sequences were subjected to pairwise paralogue comparisons to evaluate their divergence and suitability for gene-specific primer design.

4.24. Defensin-A-like

Among the antimicrobial peptide (AMP) candidates selected for further analysis was defensin-A-like gene in *Hermetia illucens* (NCBI Reference Sequence: XM_038070404.1, [LOC119661185]).

To confirm its antimicrobial potential, the full nucleotide sequence was translated using the ExPASy Translate Tool (Gasteiger, et al., 2003). The output produced six potential open reading frames (ORFs) by translating all three forward and three reverse reading directions. From these, the longest uninterrupted 5'3' frame beginning with a canonical methionine (M) and lacking internal stop codons was selected for downstream AMP and secretion prediction analyses (Figure 4.2).

The AMP Scanner v2 prediction tool classified the translated sequence as an antimicrobial peptide with a high probability score of 0.9999. This high confidence value exceeded the 0.5 classification threshold, strongly supporting its functional identity as an AMP (Figure 4.3) (Veltri, Kamath, & Shehu, 2018).

To further evaluate its secretion potential, SignalP-5.0 was used to assess the presence of a signal peptide (Nielsen, Engelbrecht, Brunak, & Von Heijne, 1997). The tool predicted a classical Sec/SPI signal with a cleavage site between positions 19 and 20, indicating that the protein is secreted via the eukaryotic secretory pathway. The prediction confidence for signal peptide classification was 0.9955, while the likelihood for classification as a non-secretory protein was only 0.0045 (Figure 4.4; Table 4.1). This result aligns with typical insect defensins, which are secreted and function extracellularly

ProP (settings: general PC = any convertase; furin-specific = just furin) returned a single high-confidence RAKR site at R58 (score 0.589) (Table 4.2; Figure 4.5) (Duckert, Brunak, & Blom, 2004). By the standard convertase convention (R-X-[K/R]-R↓), cleavage occurs C-terminal to the last Arg, so the mature defensin begins at the Ala immediately after RAKR (ATCDLLSPFGVGHAACAVHCIAMGRRGGWCDDRAVCNCRR; precursor residue 59) (Remacle, et al., 2008). Although these are in silico predictions, they inform primer placement, guide downstream expression analyses, and prioritise candidate selection.



Figure 4.2 Nucleotide Sequences translated with ExPASy Translate tool. The tool displays the six reading frames and highlights open reading frames (ORFs) in red. The longest 5'>3' ORF that begins with a methionine (M) with no internal stop codons codes determines the pro-protein (frame 2). The canonical RAKR furin-like cleavage motif (RAKR) was observed upstream of the putative mature peptide sequence. This region "ATCDLLSPFGVGHAACAVHCIAMGRRGGWCDDRAVCNCRR" was retained for AMP prediction and structure modelling.

Sequence ID(s)	Predicted Class	Prediction Probability
TTGCTTGTTAGTTATCAGTACAGAAAACGACAATCCAAAGAGCACAG/ ATTTAAAATTTGATTCTTAAACTAAAAGTC...	AMP	0.9999

i Probabilities > 0.5 = Predicted AMP

Figure 4.3 AMP prediction results for defensin-A-like using AMP Scanner v2. The nucleotide sequence was classified as an antimicrobial peptide (AMP) with a prediction probability of 0.9999, exceeding the classification threshold of 0.5.

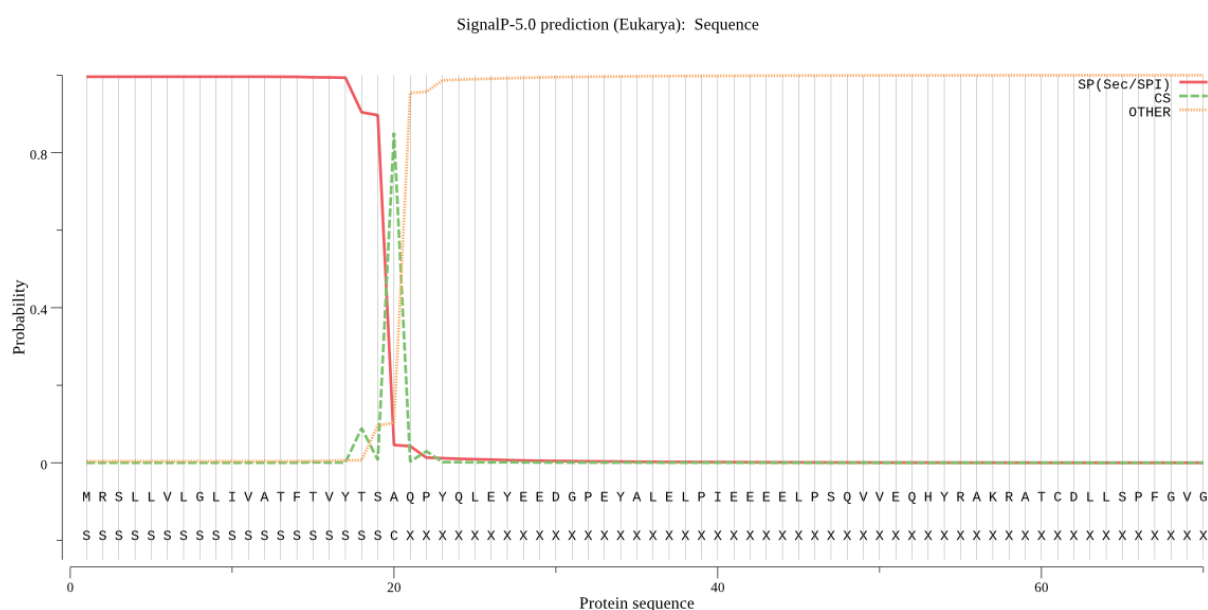


Figure 4.4 Graphical output from SignalP-5.0 showing the predicted N-terminal signal peptide in the defensin-A-like amino acid sequence. The red line represents the model's predicted probability of a Sec/SPI-type N-terminal signal peptide, peaking strongly at the N-terminus. A cleavage site is predicted between positions 19 and 20 shown by the green dashed line. The orange line indicates classification as a non-secretory protein (Other), which remained low throughout.

Table 4.1 SignalP-5.0 prediction probabilities for defensin-A-like. The model strongly predicted the presence of a classical Sec/SPI signal peptide with a likelihood of 0.9955, while the probability for non-secretory classification was only 0.0045. This supports the likelihood that defensin-A-like is secreted via the canonical eukaryotic pathway.

Protein Type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.9955	0.0045

Table 4.2 ProP convertase-site prediction for the Defensin-A-like precursor. ProP (v1.0b) identifies a single above-threshold site at R58 within the motif RAKR (score 0.589; marked “ProP”). The vertical bar indicates the predicted cleavage between the terminal Arg and the following residue (RAKR↓A, shown as ...QH_YRAKR|AT...). All other basic residues (R/K at positions 2, 55, 57, 83, 84, 91, 97, 98) score below threshold and are not considered cleavage sites.

##### ProP v.1.0b ProPeptide Cleavage Site Prediction #####											
##### General PC cleavage site prediction (Arginine/Lysine residues) #####											
Sequence	2	R	0.037	(	0.001	0.037	0.098	0.012	)	.	-----MR SL
Sequence	55	R	0.068	(	0.046	0.102	0.091	0.031	)	.	VVEQH _Y R AK
Sequence	57	K	0.064	(	0.006	0.095	0.093	0.061	)	.	EQHYRAK RA
Sequence	58	R	0.589	(	0.340	0.412	0.854	0.751	)	*ProP*	QH _Y RAKR AT
Sequence	83	R	0.041	(	0.007	0.054	0.090	0.012	)	.	HCIAMGR RG
Sequence	84	R	0.038	(	0.024	0.074	0.046	0.009	)	.	CIAMGRR GG
Sequence	91	R	0.051	(	0.004	0.067	0.076	0.055	)	.	GGWCDDR AV
Sequence	97	R	0.031	(	0.000	0.036	0.069	0.019	)	.	RAVCNCR R-
Sequence	98	R	0.051	(	0.006	0.098	0.097	0.004	)	.	AVCNCR --

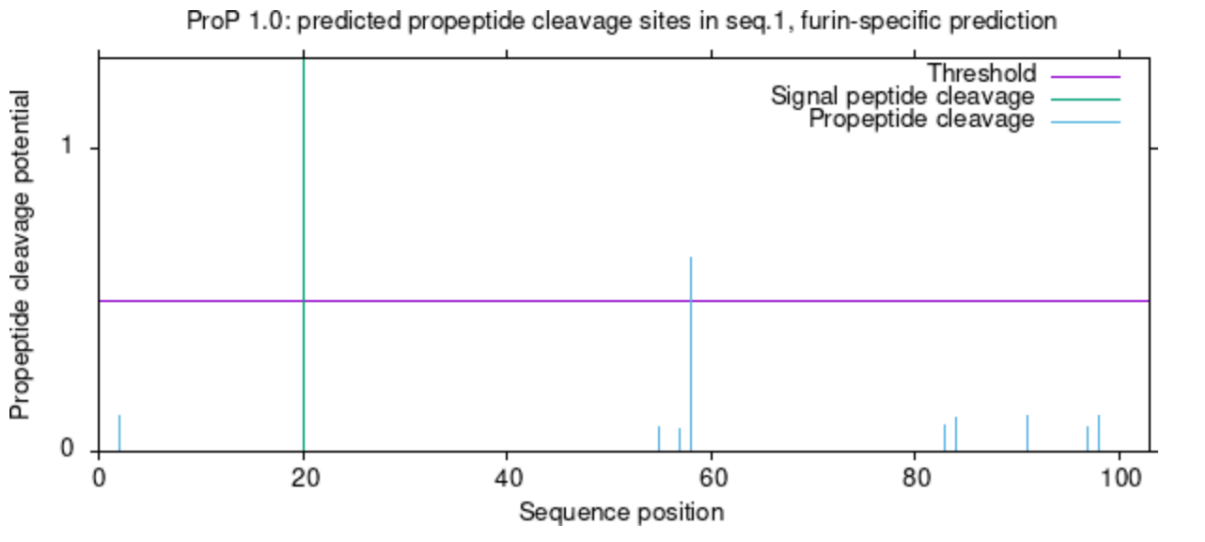


Figure 4.5 ProP (furin-specific) predictions for the Defensin-A-like precursor. Blue lines mark candidate propeptide cleavages along the precursor; the pink line is the default score threshold and the green line (~19–20) marks the SignalP signal-peptidase cut. A single above-threshold peak at ~R58 corresponds to an R-X-K-R↓ (RAKR) motif, indicating the mature peptide starts immediately after this site (A59).

To visualise the tertiary structure of the predicted mature antimicrobial domain of defensin-A-like, the protein sequence downstream of the conserved RAKR cleavage motif was submitted to ColabFold (AlphaFold2 v1.5.5 using MMseqs2 and HHsearch) (Kim, et al., 2025) (Mirdita, et al., 2022). Structural prediction produced a characteristic cis-defensin, by the presence of a α -helical and β -sheets, seen on Figure 4.6, but also by specific disulfide bond arrangement (not shown) known as the cysteine-stabilised ab motif (CSab) which follows the pattern: C1-C4, C2-C5, and C3-C6 (Bulet, Stöcklin, & Menin, 2004) (Cornet B. , et al., 1995) (Koehbach, 2017). Per-residue confidence scores

were visualised using the pLDDT colour scale. The N-terminal region exhibited moderate structural variability (pLDDT ~60–70), shown in yellow-green, whereas the central and C-terminal domains were modelled with high to very high confidence (pLDDT >90), indicated in dark blue. This suggests reliable prediction of the protein's core scaffold, particularly the β -sheet-rich regions associated with antimicrobial activity (Zhao, Lin, Yang, Ye, & Li, 2016). The spatial arrangement and conserved cysteine residues support correct disulphide bridge formation, which stabilises defensin structure and function. These features collectively reinforce the classification of this sequence as a functional antimicrobial peptide.

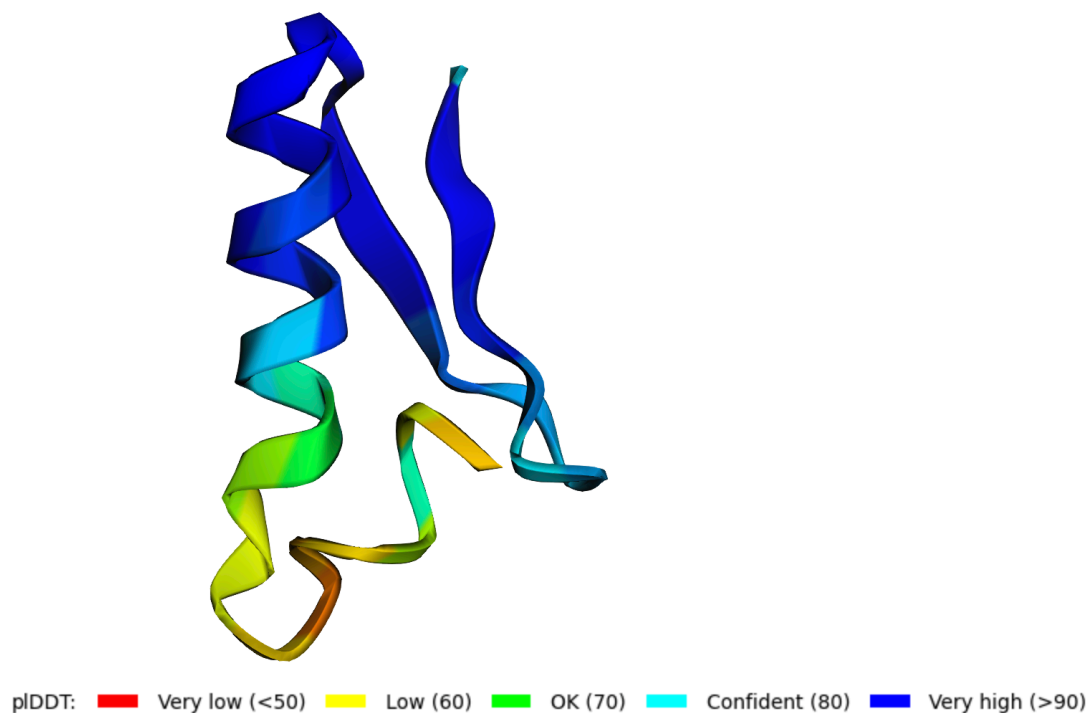


Figure 4.6 Predicted tertiary structure of the mature Defensin-A-like peptide from *Hermetia illucens* generated using AlphaFold2 via ColabFold v1.5.5. The structure is coloured by per-residue pLDDT confidence scores: red indicates very low (<50), yellow to green represent low to moderate confidence (60–70), light blue indicates confident predictions (80), and dark blue denotes very high confidence (>90). The model displays a compact α/β fold characteristic of insect defensins, with strong confidence in the core structural regions.

4.25. Defensin-B-like

To evaluate the defensin-B-like AMP, the full nucleotide sequence was translated with the ExPASy Translate tool. As with the A-like paralogue, six ORFs were returned (three forward and three reverse). The longest uninterrupted 5'>3' frame that began with a canonical methionine (M) and contained no internal stop codons was selected for downstream analyses (Figure 4.7).

AMP candidacy was then assessed with AMP Scanner v2, which classified the translated sequence as an antimicrobial peptide with maximal confidence (prediction probability = 1.000), comfortably above the 0.5 decision threshold (Figure 4.8).

Secretory routing was tested using SignalP-5.0 (Eukarya mode). The model predicted a classical Sec/SPI signal peptide with high confidence (likelihood = 0.9945; “Other” = 0.0055), and a cleavage window at the N-terminus around positions ~19–20, consistent with secretion via the canonical eukaryotic pathway (Figure 4.9; Table 4.3).

ProP identifies a single high-confidence convertase motif at R58 (RTKR), and by the standard R-X-[K/R]-R↓ convention the mature peptide begins at A59, yielding “AMCDLLSGLNMGRSVCAMRCILKGHRGGWCDDQGVNCNCRV” for structural analyses (Table 4.4; Figure 4.10). This mirrors the processing logic seen in the Defensin-A-like paralogue (RXXR-type site)

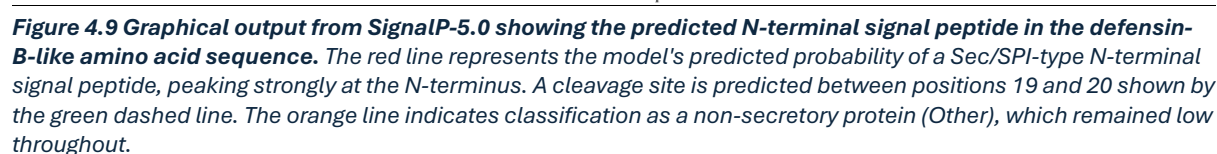


Figure 4.7 Nucleotide Sequences translated with ExPASy Translate tool. The tool displays the six reading frames and highlights open reading frames (ORFs) in red. The longest 5'>3' ORF that begins with a methionine (M) with no internal stop codons codes determines the pro-protein (frame 2). The pro-peptide cleavage motif in that precursor is RTKR, i.e., an R-X-[K/R]-R site. This region "AMCDLLSGLNMGRSVCAMRCILKGHRGWCCDDQGVNCNRV" was retained for AMP prediction and structure modelling.

Sequence ID(s)	Predicted Class	Prediction Probability
CGACAATCCACAGAACACAGATTAAGCTCCAGCTTCTTCTTCAATTTAA/ GTCAAAATGCGTTCTATTCTCGTCTTGGGT...	AMP	1.0

i Probabilities > 0.5 = Predicted AMP

Figure 4.8 AMP prediction results for defensin-B-like using AMP Scanner v2. The nucleotide sequence was classified as an antimicrobial peptide (AMP) with a prediction probability of 1.0, exceeding the classification threshold of 0.5.



Protein Type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.9945	0.0055

Name	Pos	Context	Score	Pred	
seq.1	2	-----MR	SI	0.128	.
seq.1	33	YEEDGPR	YA	0.079	.
seq.1	55	VVEQHYP	TK	0.079	.
seq.1	57	EQHYRTK	RA	0.071	.
seq.1	58	QHRYTKR	AM	0.623	*ProP*
seq.1	71	SGLNMGR	SV	0.193	.
seq.1	77	RSVCAMR	CI	0.128	.
seq.1	81	AMRCILK	GH	0.062	.
seq.1	84	CILKGHR	GG	0.067	.
seq.1	97	QGVNCNR	V-	0.080	.

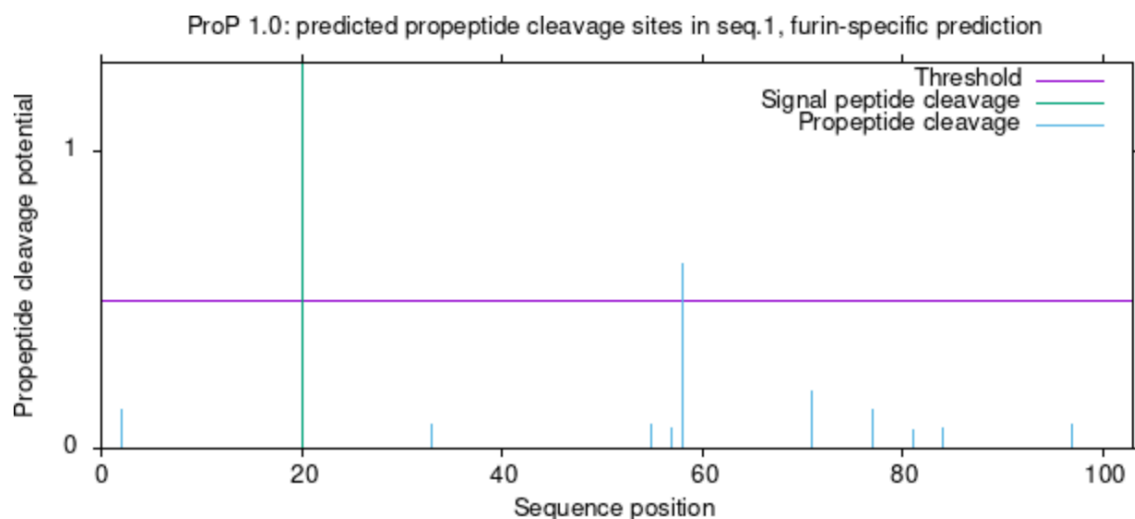
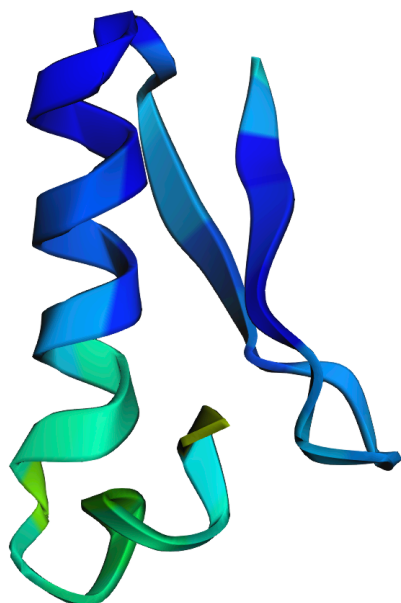


Figure 4.10 ProP prediction of convertase sites in Defensin-B-like (furin-specific mode). Blue ticks show candidate proprotein-convertase cleavages along the precursor; the pink line is the default score threshold; the green line (~19–20) marks the SignalP signal-peptidase cut. A single above-threshold peak at ~R58 corresponds to the RAKR motif (RAKR↓), designating the mature peptide start immediately after the last Arg.

AlphaFold2 was used via ColabFold v1.5.5 to predict the 3D structure of the mature peptide. The resulting model (Figure 4.11) revealed a compact α/β fold consistent with typical insect defensins. The structural confidence was evaluated using per-residue pLDDT scores, colour-coded on the model. Most of the mature domain was predicted with high confidence, particularly in core structural regions, with confidence values predominantly above 80 (light blue to dark blue). The C-terminal β -sheet and central helical regions were especially stable. A small proportion of loop regions displayed lower confidence (yellow-green), but remained within an acceptable range for predictive modelling.



pLDDT: ■ Very low (<50) ■ Low (60) ■ OK (70) ■ Confident (80) ■ Very high (>90)

Figure 4.11 Predicted tertiary structure of the mature Defensin-B-like peptide from *Hermetia illucens* generated using AlphaFold2 via ColabFold v1.5.5. The structure is coloured by per-residue pLDDT confidence scores: red indicates very low (<50), yellow to green represent low to moderate confidence (60–70), light blue indicates confident predictions (80), and dark blue denotes very high confidence (>90). The model displays a compact α/β fold characteristic of insect defensins, with strong confidence in the core structural regions.

4.26. Defensin-like-peptide 1

To assess the antimicrobial candidacy of defensin-like-peptide 1, the full nucleotide sequence was translated using the ExPASy Translate Tool. Of the six open reading frames (ORFs), 5'>3' Frame 1 was selected for AMP and secretion prediction, starting with the expected canonical methionine (M) (Figure 4.12).

The translated amino acid sequence was submitted to AMP Scanner v2, which classified it as an antimicrobial peptide (AMP) with a high prediction probability of 0.9996, strongly supporting its functional annotation as an AMP (Figure 4.13). This value exceeded the model's 0.5 classification threshold, consistent with previous defensin paralogues.

Secretory potential was then analysed using SignalP-5.0 (Eukarya mode). A classical Sec/SPI-type signal peptide was predicted at the N-terminus, with a cleavage site

between positions 19 and 20 (Figure 4.14). The likelihood for signal peptide classification was 0.9936, while the probability of being non-secretory was only 0.0064 (Table 5), reinforcing secretion via the eukaryotic secretory pathway.

ProP (both general PC and furin-specific modes) identified one confident convertase site at R58 within a RAKR motif (scores ~0.64), with cleavage C-terminal to the last Arg (RAKR↓) (Table 4.6; Figure 4.15). Accordingly, we assign the mature DLP1 to begin at A59, i.e., the region immediately downstream of RAKR (ATCDLLSPFKVGHAACALHCIALGRRGGWCDGRAVCNCRR), which was used for structural modelling and downstream analyses.



Figure 4.12 Nucleotide Sequences translated with ExPASy Translate tool. The tool displays the six reading frames and highlights open reading frames (ORFs) in red. The longest 5' to 3' ORF that begins with a methionine (M) with no internal stop codons codes determines the pro-protein (frame 1), partial base pair (RAKR) was observed upstream of the putative mature peptide sequence. This region "ATCDLLSPFKVGHAACALHCIALGRRGGWCDGRAVCNCRR" was retained for AMP prediction and structure modelling

Name	Pos	Context	Score	Pred
		v		
seq.1	2	-----MR	SV 0.173	.
seq.1	55	VVEQHYP	AK 0.082	.
seq.1	57	EQHYRAK	RA 0.076	.
seq.1	58	QHYRAKR	AT 0.638	*ProP*
seq.1	68	DLLSPFK	VG 0.055	.
seq.1	83	HCIALGR	RG 0.090	.
seq.1	84	CIALGRR	GG 0.106	.
seq.1	91	GGWCDGR	AV 0.122	.
seq.1	97	RAVCNCR	R- 0.083	.
seq.1	98	AVCNCRR	-- 0.119	.
		^		

Table 4.6 ProP calls for Defensin-like-peptide 1. For each basic residue, ProP reports the position, local sequence context (vertical bar = predicted cut), score, and decision. A single above-threshold site is detected at R58 (score 0.638, flagged ProP) with context QHYRAKR|AT, i.e., an R-X-K/R-R↓ motif.

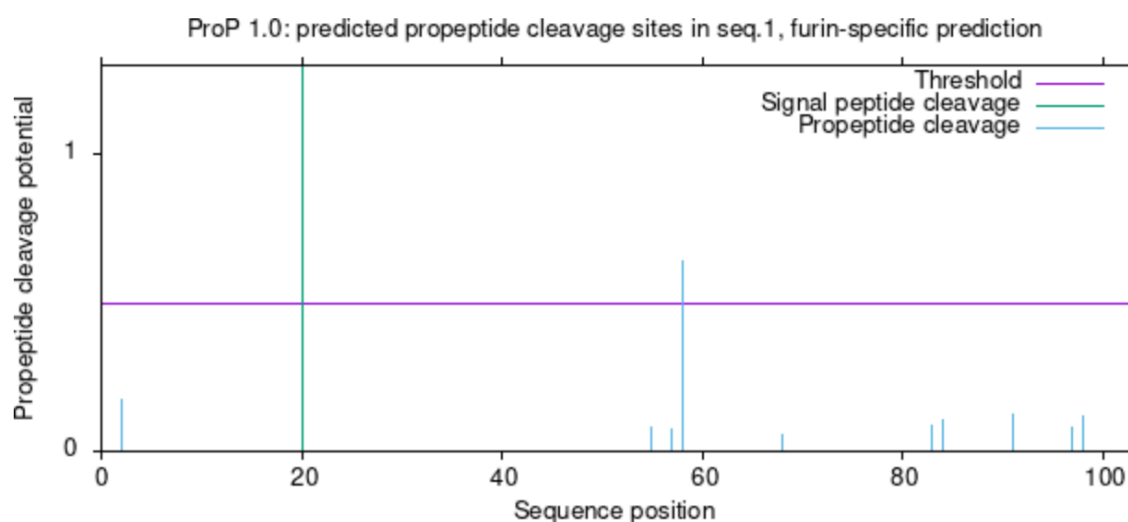


Figure 4.15 ProP (furin-specific mode) predictions for Defensin-like-peptide. Blue lines mark candidate proprotein-convertase cleavages; the pink line is the default score threshold; the green line (~20/21) marks the SignalP signal-peptidase cut. A single peak above threshold at ~58 corresponds to RAKR↓, indicating the likely mature peptide start immediately after this motif.

The mature region of defensin-like-peptide 1 was submitted to AlphaFold2 via ColabFold v1.5.5 to predict its tertiary structure. As expected, and similar to previous paralogues, we can see a tightly folded α/β motif consistent with the canonical architecture of insect defensins (Figure 4.16).

Most of the peptide exhibited high per-residue confidence, particularly in the helical core and antiparallel β -sheet domains. Confidence scores consistently exceeded 80, indicating reliable prediction accuracy in structurally important regions. Lower-confidence scores (60–70) were confined to short loop regions, a common feature in small disulphide-rich peptides, and remained within acceptable limits for in silico modelling.

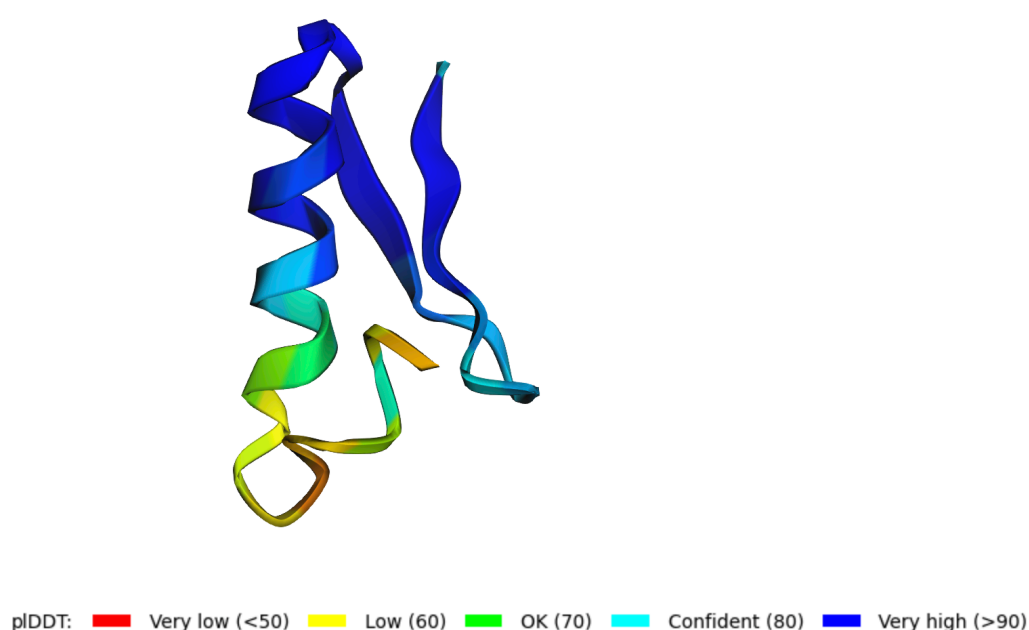


Figure 4.16 Predicted tertiary structure of the mature *Defensin-like-peptide 1* from *Hermetia illucens* generated using AlphaFold2 via ColabFold v1.5.5. The structure is coloured by per-residue pLDDT confidence scores: red indicates very low (<50), yellow to green represent low to moderate confidence (60–70), light blue indicates confident predictions (80), and dark blue denotes very high confidence (>90). The model displays a compact α/β fold characteristic of insect defensins, with strong confidence in the core structural regions.

4.27. In-silico Characterisation of *H. illucens* Defensins

Taken together, the three *H. illucens* defensins tell a very consistent story. Each transcript translates to a plausible secreted precursor: SignalP-5.0 identifies a strong Sec/SPI signal peptide at the extreme N-terminus in all cases, with a cleavage window around residues ~19–20 and near-certain probabilities. Independently, AMP Scanner v2 classifies every sequence as antimicrobial with maximal or near-maximal confidence,

reinforcing that these precursors encode bona fide AMPs rather than unrelated secreted proteins.

Downstream of the signal peptide, all three precursors converge on the same processing logic. ProP returns a single, prominent convertase site at ~R58 in every case, with the local motif matching the furin-like consensus R-X-[K/R]-R. Defensin-A-like presents RAKR, Defensin-B-like presents RTKR, and Defensin-like-peptide 1 (DLP1) again presents RAKR; in each instance the predicted cut lies C-terminal to the final Arg, so the mature peptide begins at the following Ala (A59). Thus, despite modest motif variation between paralogues, the position of the inferred pro-region/mature boundary is essentially co-linear across the family, yielding mature peptides 40 aa (amino acids) in length.

ColabFold/AlphaFold2 models of the post-RXXR segments produce the canonical CS α β defensin architecture, a compact α -helix packed against a short antiparallel β -sheet, with high pLDDT confidence in the helical core and β -sheet and only modest uncertainty confined to terminal loops. The combination of secretion signals, a shared RXXR processing site, and a convergent defensin fold strongly supports the functional annotation of Defensin-A-like, Defensin-B-like, and DLP1 as secreted defensin-type AMPs. While these boundaries are predicted rather than experimentally mapped, the internal consistency across tools and paralogues provides a solid working model for the rest of the study. In practice, these calls guide primer placement, frame downstream expression analyses, and prioritise candidates for follow-up. We explicitly treat the mature-peptide starts and disulfide connectivities as putative, with targeted proteomics (e.g., N-terminomics/LC-MS) and structural validation reserved to confirm the precise cleavage positions and bonding patterns.

4.28. Parologue Validation of Defensin AMP Targets

To confirm that the selected defensin targets represented distinct paralogues and to validate primer specificity, pairwise nucleotide alignments were performed using Alignment in Clustal Omega (Figure 4.17) and NCBI BLAST2 (Table 4.7) (Zheng, Scott,

Lukas, & Webb, 2000), (Aleksandr, et al., 2008). Comparisons across defensin-A-like, Defensin-B-like, and Defensin-like peptide 1 revealed moderate sequence divergence, consistent with their classification as separate but related genes.

Defensin-A-like and Defensin-B-like shared 81.68% nucleotide identity across 76% of the query sequence, with an alignment length of 393 bp and a significant E-value of $2e^{-110}$. In contrast, defensin-A-like and defensin-like-peptide 1 exhibited a slightly higher identity of 83.61% and broader coverage (80%, 514 bp), supported by the highest alignment scores among the comparisons (max score: 432). The closest paralogues were defensin-A-like and defensin-like-peptide 1, sharing 81.89% identity with 97% query coverage across a 514 bp region ($E = 2e^{-105}$), suggesting a higher degree of overlap but still sufficient sequence variation to allow specific discrimination.

```

CAGAAAACGACAATCCAAAGAGCACAGAT 70 80 90 100 110 120
TTAAGCTCCAGCTTCTTCTCAATTTAAA ATTGTTGGCACTTTCAAGGTCTACACCTCAGCAACCAATATCAGTAATAACGAGAA
CTGCTAGAATACAGATCCAGCTTCAGCTT ATTGTTGGCACTTTTGTGTCTACACCTCAGCAACCAATATCAGTAATAACGAGAA

CAGAAAACGACAATCCAAAGAGCACAGAT 130 140 150 160 170 180
TTAAGCTCCAGCTTCTTCTCAATTTAAA GATGGTCTGAATACGCACTGGAACCTCTATTGAAGAAGAAGAACTTCTAGTCAGGTAA
CTGCTAGAATACAGATCCAGCTTCAGCTT GATGGTCTAGATACGCACTGGAACCTCTATTGAAGAAGAAAGCGCTTCTGTCAGGTG
GATGGTCTCGATCAGCACTGGAACCTCTATTGAAGAAGAACAACTTCTGAGTCAGGTG

CAGAAAACGACAATCCAAAGAGCACAGAT 190 200 210 220 230 240
TTAAGCTCCAGCTTCTTCTCAATTTAAA GTGGAGCAGCATTATCGGGCAAAACGTGCAACCTGTGATCTCTTGAGCCCTTCGGCGTGA
CTGCTAGAATACAGATCCAGCTTCAGCTT GTGGAGCAGCATTATCGGGCAAAACGTGCAATGTGTGATTTATTGAGTGGCTTGAAACATG
GTGGAGCAGCATTATCGTGC AAAACGTGCAACCTGTGATCTCTTGAGTCCCTTCAAACTG

CAGAAAACGACAATCCAAAGAGCACAGAT 250 260 270 280 290 300
TTAAGCTCCAGCTTCTTCTCAATTTAAA GGTCACTGGCGCTGCGCATTTCAATTGTATGGCATGGGACGACGGAGGATGGTGCGGAT
CTGCTAGAATACAGATCCAGCTTCAGCTT GGTCACTGGCGCTGCGCATTTCAATTGTATCCTTAAAGGACATCGGGAGGCTGGTGCGGAT
GGTCACTGGCGCTGCGCATTTCAATTGTATGGCTTTGGGACGTCTGGAGGCTGGTGCGGAT

CAGAAAACGACAATCCAAAGAGCACAGAT 310 320 330 340 350 360
TTAAGCTCCAGCTTCTTCTCAATTTAAA GACCGAGCCGTTTGTAATTGCAGACGTTAATCTAAAGTGCTTATATATATATAGAGCTC
CTGCTAGAATACAGATCCAGCTTCAGCTT GGTCACTGGCGCTGCGCATTTCAATTGTATCTAATCTAAAGTGCTTATATATATAAACAACAC

CAGAAAACGACAATCCAAAGAGCACAGAT 370 380 390 400 410 420
TTAAGCTCCAGCTTCTTCTCAATTTAAA CAATTTTTTATTATTATTTACCAATTTTGTAAATTTATCAATTGTTAATTGTTGTTATAA
CTGCTAGAATACAGATCCAGCTTCAGCTT CAATTTGTTATTATTATTCACAAATTTTATTAGTAGTTGTTATTTTGGTTAAAAAATT

CAGAAAACGACAATCCAAAGAGCACAGAT 430 440
TTAAGCTCCAGCTTCTTCTCAATTTAAA ATAAAAATTTCTAAATTC.....GGGAAAA..
CTGCTAGAATACAGATCCAGCTTCAGCTT ATTTGGGAAAAATAATTATTATTTTTTTTTTAAATTG

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Figure 4.17 Alignment in CLUSTAL format with base/residue numbering. Sequence alignment analysis between Defensin-A-like, Defensin-B-like, and Defensin-like-peptide 1, generated by Clustal Omega (Sievers, 2014) and graphics generated by EndScript 2 (Robert & Gouet, 2014)

Table 4.7 Pairwise nucleotide alignment of three defensin paralogues (defensin-A-like, defensin-B-like, and Defensin-like-peptide 1). Sequences were aligned using NCBI BLAST2 to assess nucleotide identity, query coverage, alignment length, and alignment scores. Moderate identity values confirm that each gene represents a distinct but closely related paralogue suitable for target-specific primer design.

Paralogue Comparisons	% Identity	Query cover	Alignment length	E-value	Max/Total score
Defensin-A-like vs Defensin-B-like	81.68%	76%	393	2e-110	381/406
Defensin-A-like vs Defensin-like peptide 1	83.61%	80%	514	2e-125	432/432
Defensin-B-like vs Defensin-like-peptide 1	81.89%	97%	514	2e-105	366/390

4.29. Attacin AMP Selected For Transcript Screening

Our next screening of Antimicrobial peptides were for Attacins, two of which were selected for analysis. These included two paralogues of attacin-A-like (Hill_BB_C16882|attacin and Hill_BB_C10074|attacin-like), both obtained from an available *Hermetia illucens* transcriptome (Vogel H. , Müller, Heckel, Gutzeit, & Vilcinskis, 2018).

We looked at the first paralogue of attacin-A-like gene and translated the full nucleotide sequence in ExPASy Translate Tool (Figure 4.18). Unlike defensins, where conserved furin cleavage motifs such as RAKR or KRA are frequently used to define the boundary of the mature peptide, no canonical processing motif was observed in this Attacin-A-like sequence. Therefore, the mature peptide region was defined by the predicted signal peptide cleavage site at AA20.

Antimicrobial classification was then assessed using AMP Scanner v2. The translated protein was classified with a maximum prediction probability of 1.000, indicating strong likelihood of antimicrobial functionality (Figure 4.19). Like before, this prediction exceeds the default classification threshold of 0.5 and supports annotation of the sequence as a putative AMP.

To determine whether the peptide is secreted, SignalP-5.0 (Eukarya mode) was used to evaluate the presence of an N-terminal signal peptide. The model strongly predicted a classical Sec/SPI secretion signal with a confidence of 0.927, while the probability of

non-secretory classification was only 0.073 (Table 4.8). A cleavage site was predicted between residues 19 and 20 of the amino acid sequence (Figure 4.20), aligning with the typical structure of insect attacins, which are known to be secreted via the eukaryotic pathway.

Unlike the defensins, ProP (general PC and furin-specific) detects no above-threshold (K/R)xn(K/R) convertase sites in the downstream region; there is no RXXR motif in this ORF, and only sub-threshold dibasic residues are present (Table 4.9; Figure 4.21). Consequently, we adopt a conservative boundary and retain the post-SignalP sequence (“QLSGSITPDMAGGNNVNIMASKFLGNPNHNIGGGVFAAGNTRSNTPSLGAFGTLNLKDHSLGVSKTITPGVSDTFSQNARLNILKTPDHRVDANVFNSHTRLNNGFAFDKRGGSLDYTHRAGHGLSLGASHIPKFGTTAELTGKANLWRSPSGLSTFDLTGSASRTFGGPMAGRNNFGAGLGFSHRF”) as the working secreted product for all downstream analyses. This outcome may be consistent with reported diversity among attacins, such as in *Drosophila* where some paralogues lack a pro-peptide (Qi, Gao, & Zhu, 2021).

4.30. Attacin-A-like paralogue 1



Figure 4.18 Nucleotide Sequences translated with ExPASy Translate tool. The tool displays the six reading frames and highlights open reading frames (ORFs) in red. The longest 5' to 3' ORF that begins with a methionine (M) with no internal stop codons codes determines the pro-protein (frame 3), partial base pair (RAKR) was observed upstream of the putative mature peptide sequence. The region at the 20th amino acid predicted by SignalP5.0 “QLSGSITPDMAGGNNVNIMASKFLGNPNHNIGGGVFAAGNTRSNTPSLGAFGTNLNLDHSLGVSKTITPGVSDTFSQARLNILKTPDHRVDANVFNHSHTRLNNGFAFDKRGSLDYTHRAGHGLSLGASHIPKFGTTAELTGKANLWRSPLSGSTFDLTGSASRTFGPMAGRNNFGAGLGFSHRF” was retained for AMP prediction and structure modelling

Sequence ID(s)	Predicted Class	Prediction Probability
AATTATTCACTTATTAGTATTAACCTAACGAGGAAAGTATTTGTATTAA AAGGACATCGATGATTATTGCAATTGTTGC...	AMP	1.0

i Probabilities > 0.5 = Predicted AMP

Figure 4.19 AMP prediction results for Attacin-A-like using AMP Scanner v2. The nucleotide sequence was classified as an antimicrobial peptide (AMP) with a prediction probability of 1, exceeding the classification threshold of 0.5.

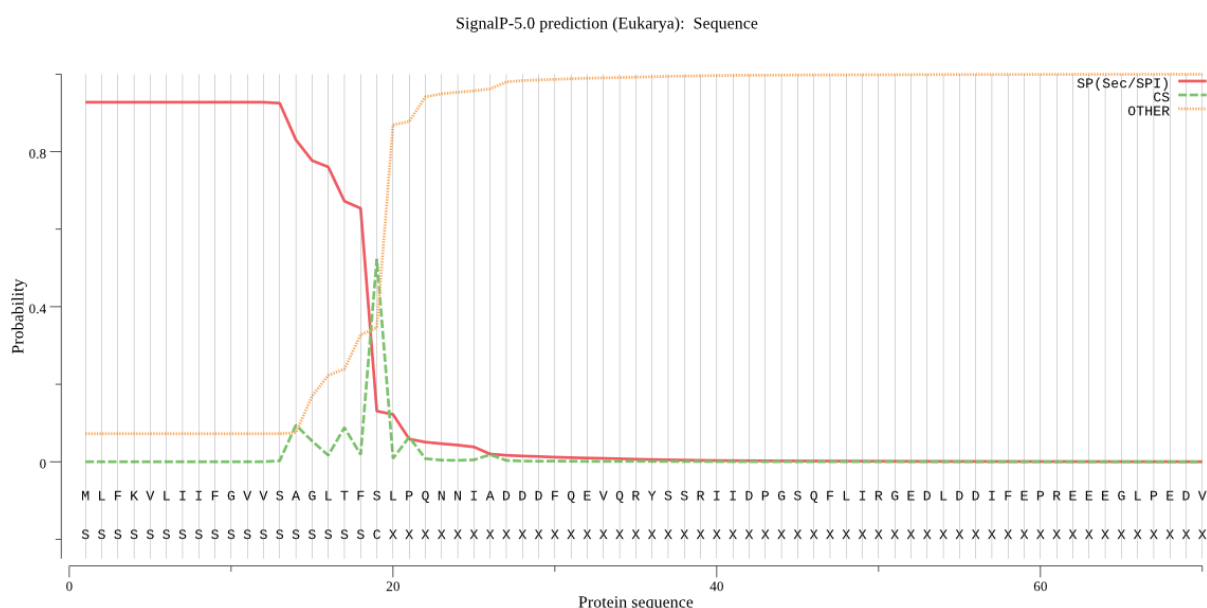


Figure 4.20 Graphical output from SignalP-5.0 showing the predicted N-terminal signal peptide of the first Attacin-A-like paralogue. The red line represents the model's predicted probability of a Sec/SPI-type N-terminal signal peptide, peaking strongly at the N-terminus. A cleavage site is predicted between positions 19 and 20 shown by the green dashed line. The orange line indicates classification as a non-secretory protein (Other), which remained low throughout.

Table 4.8 SignalP-5.0 prediction probabilities for Attacin-A-like. The model strongly predicted the presence of a classical Sec/SPI signal peptide with a likelihood of 0.927, while the probability for non-secretory classification was only 0.073. This supports the likelihood that the first Attacin-A-like paralogue is secreted via the canonical eukaryotic pathway.

Protein Type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.927	0.073

Name	Pos	Context	Score	Pred
		v		
Sequence	5	--MALTR	TS 0.081	.
Sequence	44	VNIMASK	FL 0.077	.
Sequence	64	FAAGNTR	SN 0.152	.
Sequence	79	FGTNLK	DH 0.057	.
Sequence	87	HSLGVSK	TI 0.071	.
Sequence	102	TFSQNAR	LN 0.103	.
Sequence	107	ARLNILK	TP 0.061	.
Sequence	112	LKTPDHR	VD 0.077	.
Sequence	123	VFNSHTR	LN 0.086	.
Sequence	132	NGFAFDK	RG 0.061	.
Sequence	133	GFAFDKR	GG 0.157	.
Sequence	142	SLDYTHR	AG 0.079	.
Sequence	156	GASHIPK	FG 0.099	.
Sequence	166	TAELTGK	AN 0.060	.
Sequence	171	GKANLWR	SP 0.134	.
Sequence	187	LTGSASR	TF 0.084	.
Sequence	196	GGPMAGR	NN 0.095	.
Sequence	208	GLGFSHR	F- 0.114	.
		^		

Table 4.9 ProP (general PC mode) calls for Paralogue 1 of Attacin-A-like. For each basic residue, ProP reports position, local sequence context (vertical bar = predicted cut), score, and decision. No Arg/Lys sites exceed the default threshold (Arg/Lys sites predicted above threshold: 0), indicating no confident proprotein-convertase motif in the pro-region.

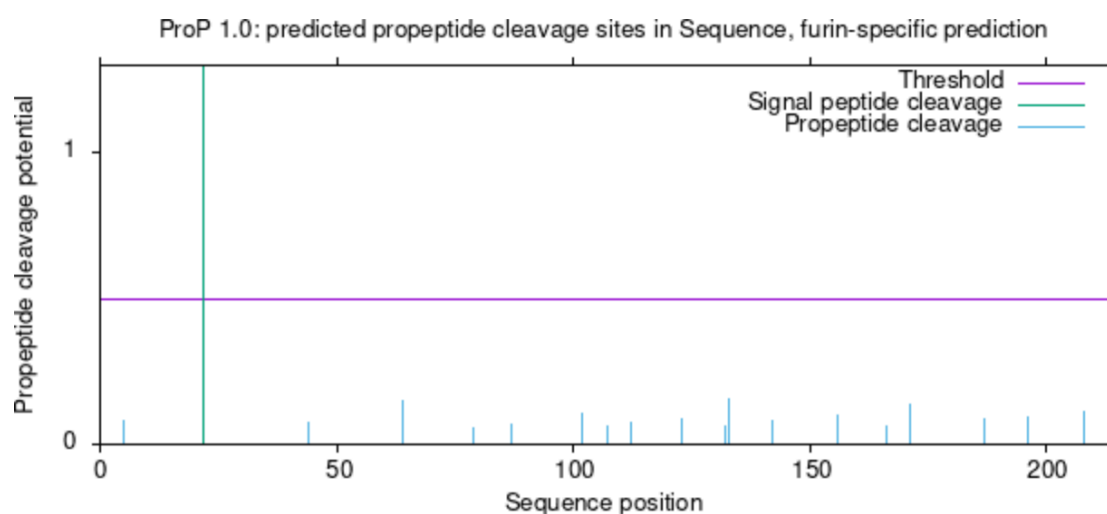


Figure 4.21 ProP (furin-specific mode) profile for Paralogue 1 of Attacin-A-like. Blue lines mark candidate convertase cleavages; the pink line is the default threshold; the green line (~22–23) marks the SignalP cut. No peaks rise above threshold, consistent with Table 9.

AlphaFold2 was used via ColabFold v1.5.5 to generate the 3D tertiary structure of the mature Attacin-A-like peptide. The predicted model revealed a highly ordered β -sheet-rich fold (Figure 4.22) and confidence prediction was high throughout the structure, with pLDDT scores >90 (dark blue) across most β -strands, and slightly lower confidence

(green-yellow) in loop regions. Compared to previous defensin models, which displayed compact α/β motifs, the Attacin-A-like paralogue adopted a more extended, barrel-like conformation, which is typical of attacin AMP folds. In contrast to the defensins, which include a short acidic pro-region ending in a furin-type R-X-[K/R]-R↓ site, the attacin-A-like precursor showed no above-threshold convertase sites in ProP. Therefore, we defined the secreted product directly from the C-terminus of the SignalP-5.0 signal-peptidase cut.

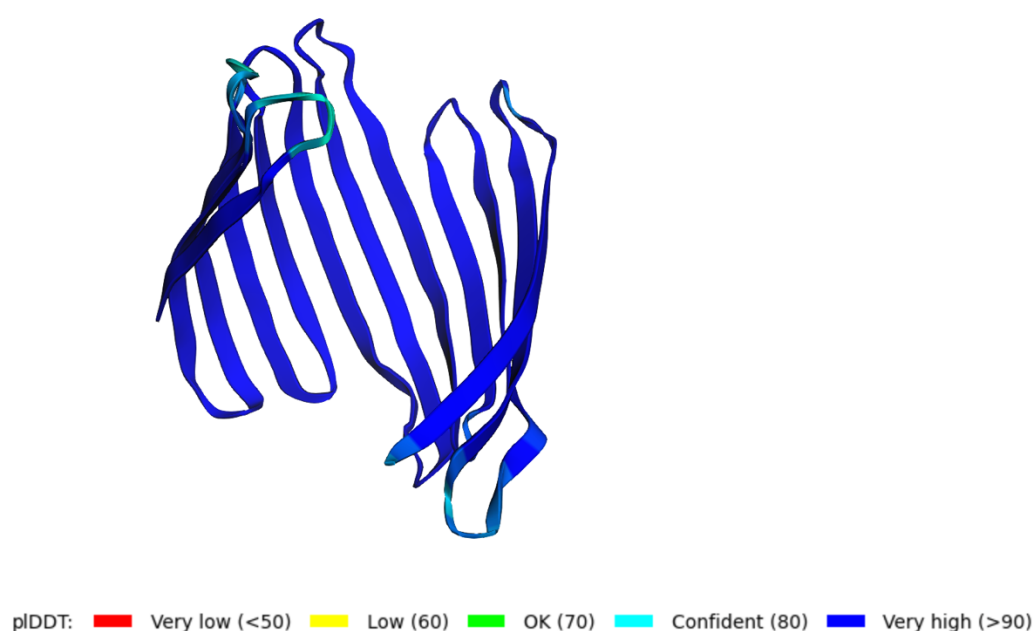


Figure 4.22 Predicted tertiary structure of the first paralogue of Attacin-A-like from *Hermetia illucens* generated using AlphaFold2 via ColabFold v1.5.5. The structure is coloured by per-residue pLDDT confidence scores: red indicates very low (<50), yellow to green represent low to moderate confidence (60–70), light blue indicates confident predictions (80), and dark blue denotes very high confidence (>90). The model displays a compact α/β fold characteristic of insect defensins, with strong confidence in the core structural regions.

4.31. Attacin-A-like Paralogue 2

The second attacin-A-like paralogue was identified from the *Hermetia illucens* transcriptome and subjected to the same in silico AMP validation pipeline as the first paralogue. We obtained the same translation from ExPASy Translate Tool to determine the longest 5'→3' ORF without internal stop codons (Figure 4.23).

AMP potential was then assessed using AMP Scanner v2. The full translated sequence was confidently classified as an antimicrobial peptide, with a prediction probability of 1.0, well above the classification threshold of 0.5 (Figure 4.24).

Secretory signal peptide detection was conducted using SignalP-5.0 in Eukarya mode. The model predicted a Sec/SPI-type signal peptide at the N-terminal region with high probability (0.927), and a very low probability for non-secretory classification (0.073) (Table 10). The predicted cleavage site occurred between residues 19 and 20, consistent with canonical secretion via the eukaryotic pathway (Figure 4.25).

In contrast to attacin-like paralogue 1 where there was the apparent absence of a cleavage motif, paralogue 2 contains a single above-threshold furin-type site (...DVIRARR↓SPQD...) at position 75, with all other arginine-rich motifs scoring below threshold. Sequence

“SPQDGRRGSASVTVNNESRRGTDVRADLNARLWEGNNRRSSLDANAYYQRHFGGPMGTG
RPDAGVGLNFRHRF” was therefore retained for downstream structure predictions.



Figure 4.23 Nucleotide Sequences translated with ExPASy Translate tool. The tool displays the six reading frames and highlights open reading frames (ORFs) in red. The longest 5' to 3' ORF that begins with a methionine (M) with no internal stop codons codes determines the pro-protein (frame 2). The region after the canonical cleavage motif RARR “SPQDGRRGASVTVNNESRRGTDVRADLNARLWEGNNRRSSLDANAYYQRHFGGPMGTGRPDAGVGLNFRHRE” was retained for AMP prediction and structure modelling

Sequence ID(s)	Predicted Class	Prediction Probability
AATTCGCAATTATTCTACTTAACAAAGCTTCGAAGAAAAGATCTTCTCAA AGTTTAATTAATCAACCAATCTTCAAGT...	AMP	1.0

i Probabilities > 0.5 = Predicted AMP

Figure 4.24 AMP prediction results for the second Attacin-A-like paralogue using AMP Scanner v2. The nucleotide sequence was classified as an antimicrobial peptide (AMP) with a prediction probability of 1, exceeding the classification threshold of 0.5.

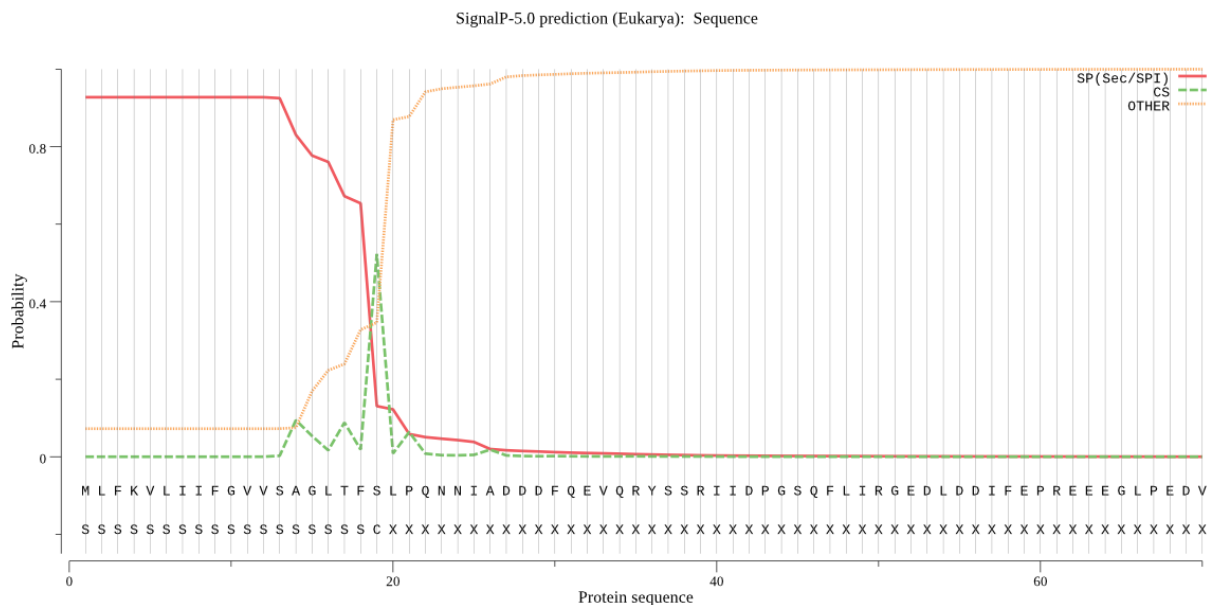


Figure 4.25 Graphical output from SignalP-5.0 showing the predicted N-terminal signal peptide of the second Attacin-A-like paralogue. The red line represents the model's predicted probability of a Sec/SPI-type N-terminal signal peptide, peaking strongly at the N-terminus. A cleavage site is predicted between positions 19 and 20 shown by the green dashed line. The orange line indicates classification as a non-secretory protein (Other), which remained low throughout

Table 4.10 SignalP-5.0 prediction probabilities for the second paralogue Attacin-A-like. The model strongly predicted the presence of a classical Sec/SPI signal peptide with a likelihood of 0.927, while the probability for non-secretory classification was only 0.073. This supports the likelihood that the first Attacin-A-like paralogue is secreted via the canonical eukaryotic pathway.

Protein Type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.927	0.073

Name	Pos	Context	Score	Pred
		v		
Sequence	4	---MLFK VL	0.060	.
Sequence	35	DFQEVQR YS	0.116	.
Sequence	39	VQRYSSR II	0.121	.
Sequence	50	GSQFLIR GE	0.079	.
Sequence	61	DDILEPR EE	0.082	.
Sequence	72	LPEDVIR AR	0.096	.
Sequence	74	EDVIRAR RS	0.082	.
Sequence	75	DVIRARR SP	0.634	*ProP*
Sequence	81	RSPQDGR RG	0.085	.
Sequence	82	SPQDGRR GS	0.158	.
Sequence	94	TVNNESR RG	0.101	.
Sequence	95	VNNESRR GT	0.188	.
Sequence	100	RRGTDVR AD	0.109	.
Sequence	106	RADLNAR LW	0.085	.
Sequence	113	LWEGNNR RS	0.073	.
Sequence	114	WEGNNRR SS	0.196	.
Sequence	125	ANAYYQR HF	0.121	.
Sequence	135	GPMGTGR PD	0.108	.
Sequence	145	GVGLNFR HR	0.078	.
Sequence	147	GLNFRHR F-	0.128	.
		^		

Table 4.11 Furin/PC convertase motif predictions for the attacin-A-like paralogue 2 ORF. One site exceeds the ProP threshold: DVIRARR|S at Pos 75 (score 0.634, flagged ProP). All other dibasic/arginine-rich sites score below threshold (no cleavage predicted).

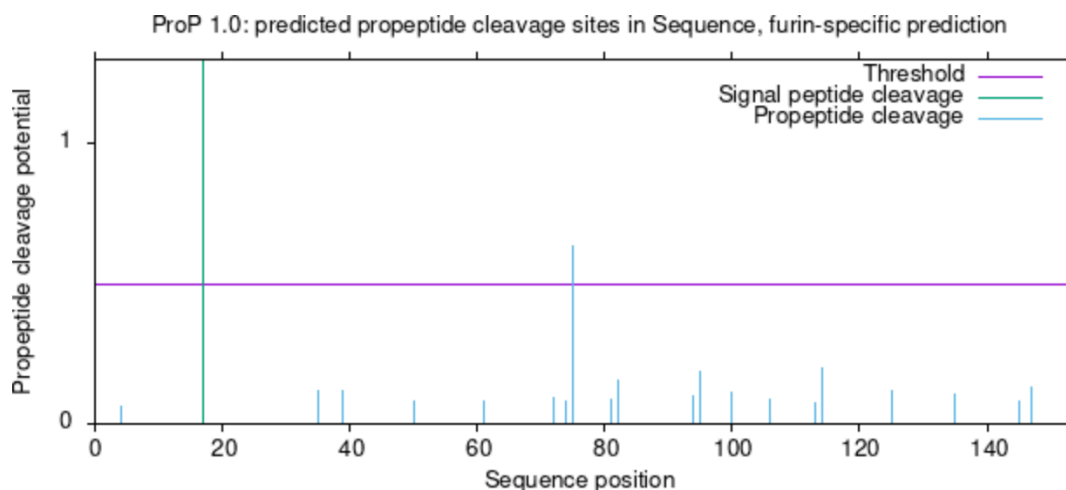


Figure 4.26 Cleavage potential across the sequence. The green vertical line marks the SignalP-5.0 signal-peptidase cut at S19|T20. Blue bars show candidate propeptide convertase sites; the prominent bar at ~75 crosses the purple threshold and corresponds to the DVIRARR↓S furin-type site. No other peaks exceed threshold.

After signal-peptide removal (S19|T20) and the predicted furin-type cleavage at DVIRARR↓S (Pos 75), the predicted mature chain (S76–end) adopts a compact β -sheet-of four anti-parallell strands dominated with high confidence across the core and a low-confidence, likely flexible terminal segment.

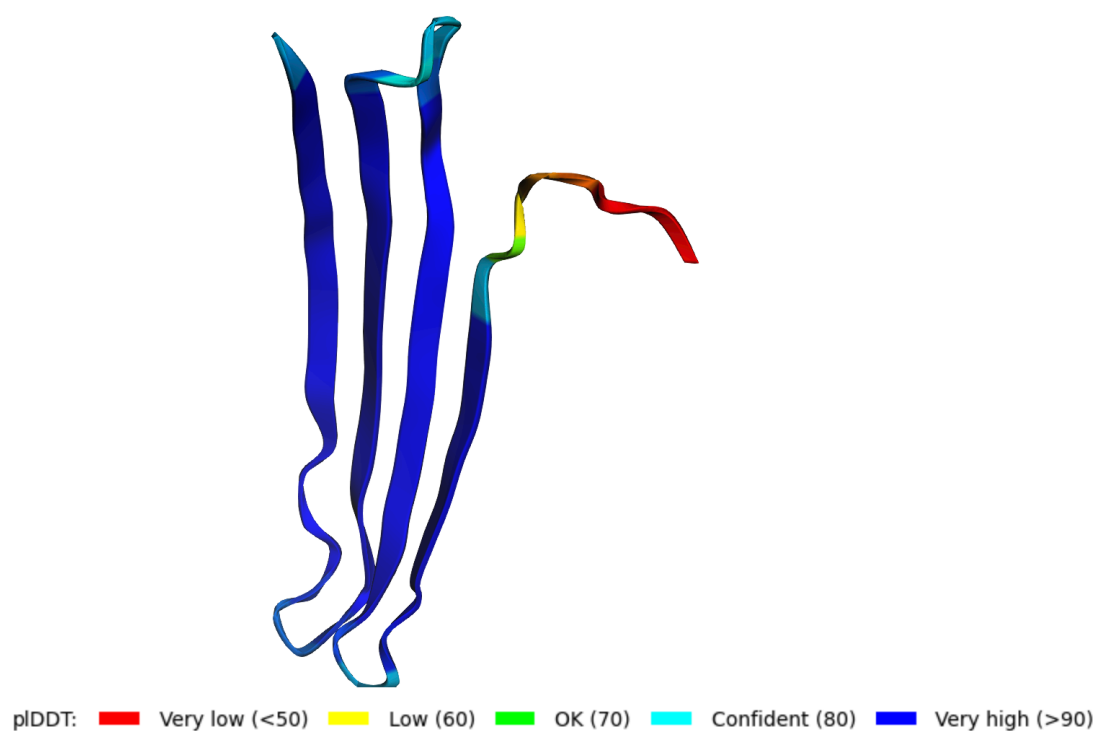


Figure 4.27 ColabFold/AlphaFold2 model of the mature Attacin-A-like paralogue 2. Ribbon is coloured by pLDDT (model confidence): blue = very high (>90), light blue = high (~80), green/yellow = moderate/low (70–60), red = very low (<50). The structure shows an elongated, β -sheet-rich core composed of four antiparallel strands with a short projecting loop/terminal tail that is modelled with low confidence (yellow/red).

4.32. Parologue Validation of Attacin AMP Targets

Similarly, Clustal omega (Figure 4.28; Table 4.29) and a pairwise comparison was performed to investigate the relationship between two attacin-like sequences identified in the *Hermetia illucens* transcriptome (Vogel, Muller, Heckel, Gutzeit, & Vilcinskas, 2018). Despite both being annotated as putative attacin paralogues, nucleotide-level alignment using BLASTn revealed no significant similarity between the sequences, suggesting high divergence or alternative evolutionary origins. To assess potential conserved protein domains, a tBLASTx alignment was conducted, which revealed low amino acid similarity (36.11% identity) across just 25% of the sequence ($E = 8e^{-7}$), indicating only marginal overlap at the translated level.

```

1      10      20      30      40      50      60
TAACCTAACGAGGAAAGTATTTTGTATTA AAGGACATCGATGATTTTCANFTGTCGATTCGGGTACTCAAATGGGCCATC
ACAAAGCTTCGAAGAAAAGATCTTCTCAA ...AGTTAATTAATCAACCAACCTTCAAGATGTT...GTCAAAGGCTCATTAT

70      80      90      100     110     120
TAACCTAACGAGGAAAGTATTTTGTATTA ATCAATGATCGATTACACCCATAGGAGGAGGCAATAATGTTAACATCATGGCATGAA
ACAAAGCTTCGAAGAAAAGATCTTCTCAA TCGCT.....CTGGCTGCGGG.....CTTA

130     140     150     160     170     180
TAACCTAACGAGGAAAGTATTTTGTATTA ATCTCGGAAACCCCATCAACATTGGTGAAGATTTCGCCGAGCAACACACG
ACAAAGCTTCGAAGAAAAGATCTTCTCAA CGTTTCGC..TGCCCAAAACAATTGCGCATATGACTTCGAAGAGTGCAAAGGT.

190     200     210     220     230
TAACCTAACGAGGAAAGTATTTTGTATTA ATCAATACCCATCTTGGGA..CTTTGGAACTTGAACCTAAAGACCTAGTCTG
ACAAAGCTTCGAAGAAAAGATCTTCTCAA ATTCATGAGATATTGACGCGCGAGTCAGTCTTGAATCGGTGGAGGACTTA..G

240     250     260     270     280     290
TAACCTAACGAGGAAAGTATTTTGTATTA GGCGCTCGGAACCAATACTCCAGGGTAAGTGAATTCATCAAAATGACCAATA
ACAAAGCTTCGAAGAAAAGATCTTCTCAA ATGAATTTTA..GAGCGGAGAGAGGAAGGTTTACCGAAGATGTAATTCGAGCGAG

300     310     320     330     340     350
TAACCTAACGAGGAAAGTATTTTGTATTA ATATCTCGAAGACTCCGATATCCATGGACCGCA..CTTTCAATAGATATCA
ACAAAGCTTCGAAGAAAAGATCTTCTCAA AGGATCACCGC..AAGAGGAGTCGCGTTTCTGCTAGTGTCACAGTAATAATCAAGC

360     370     380     390     400     410
TAACCTAACGAGGAAAGTATTTTGTATTA AGGCATATATGATTCGATTCGATTAACGAGGCGTAGTTGGATTATACCAACAGG
ACAAAGCTTCGAAGAAAAGATCTTCTCAA AGGCCTGGCACTGATACCAAGCTCATCTCAACGCTAGACTTGGGAGGTACCAATAGA

420     430     440     450     460     470
TAACCTAACGAGGAAAGTATTTTGTATTA GCAGGACATGGTCTTCTTTGCGAGCAGTCATATCCATAAATTGGGAAGCACTGCTCAA
ACAAAGCTTCGAAGAAAAGATCTTCTCAA AGGTCAAGTTTAGAGCTAATCCCTATCAACCCATTTTCGGTGGACCA...TCG.

480     490     500     510     520     530
TAACCTAACGAGGAAAGTATTTTGTATTA TTACGGGCAAAGCTAATCTCTGGATCACCCACTGTCTATCAACTTTTATATTAAC
ACAAAGCTTCGAAGAAAAGATCTTCTCAA GACCGGAC...CACAGATGCTGGAGTTGAAATCACTTTCACATTCGTTCTAGAT

540     550     560     570     580     590
TAACCTAACGAGGAAAGTATTTTGTATTA GCAATGTCATCGAGAACGTTGCGACCAATGGCGCTAAGAAATTTGGTGCAGGT
ACAAAGCTTCGAAGAAAAGATCTTCTCAA ACAT.....CGCGTAGCAGCAGCACATCATCAATTA.....

600     610     620     630     640     650
TAACCTAACGAGGAAAGTATTTTGTATTA TTAGCATTTAGCCATAAGGTTTAGTGAATCTATAAATATTCGTTATGTTAAATTA
ACAAAGCTTCGAAGAAAAGATCTTCTCAA ....ATTTATCAATTTCAATATATAA....CGCACTGTAGGTTA...AAAG

660     670     680     690     700
TAACCTAACGAGGAAAGTATTTTGTATTA TTTTAAATACAAAGTCTGGAGAAATATAATGCATAAAGATCAA
ACAAAGCTTCGAAGAAAAGATCTTCTCAA ATTTCTCTAGCAACTGA...ACAATATATTTTGCAAGATTC.

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Figure 4.28 Alignment in CLUSTAL format with base/residue numbering. Attacin sequence alignment analysis generated by Clustal Omega (Sievers, 2014) and graphics generated by EndScript 2 (Robert & Gouet, 2014)

4.33. Attacin-A-like Parologue comparisons

Table 4.12 Sequence comparison of Attacin-A-like paralogues. Pairwise alignment shows 25% query coverage, 36.1% amino-acid identity, and a significant match ($E\text{-value } 8 \times 10^{-7}$), consistent with conservation of the Attacin core region.

Sequence	Query Cover	% Identity	E-value
Comparison			
Attacin-A-like paralogues	25%	36.1%	8×10^{-7}

4.34. *Hermetia illucens* cecropin-like peptide 1

Our next antimicrobial peptide for analysis was cecropin-like peptide (Hill_SB_C698|cecropin) from *Hermetia illucens* (Vogel, Muller, Heckel, Gutzeit, & Vilcinskis, 2018).

The *Hermetia illucens* cecropin-like candidate was translated using ExPASy tools to find the longest reading ORF frame (Figure 4.30; frame 1). AMP Scanner v2 classified the nucleotide sequence as an antimicrobial peptide with a prediction probability of $P=0.9999$ (Figure 4.31). SignalP-5.0 predicts a strong Sec/SPI signal peptide (0.9953) with low score predictions for non-secretory classifications. Cleavage is predicted at SEA|GW (23|24), indicating export via the ER/Golgi pathway (Figure 4.32; Table 4.13). ProP-1.0 (furin-specific) detects no above-threshold convertase sites, predicting that, this precursor is not further processed by a canonical R-X-[K/R]-R↓ convertase. We therefore define the secreted product as the chain immediately downstream of the signal-peptidase cut (res. 24→C-terminus) “GWWKRVFKPVEKLGQRVRDAGIQGLEIAQQGANVLATARGGPPQQG” (Table 4.14; Figure 4.33).

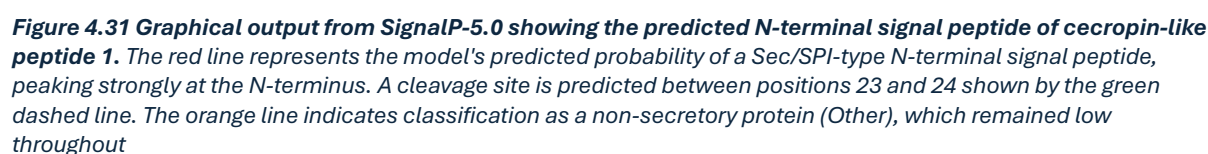


Figure 4.29 Nucleotide Sequences translated with ExPASy Translate tool. The tool displays the six reading frames and highlights open reading frames (ORFs) in red. The longest 5'>3' ORF that begins with a methionine (M) with no internal stop codons codes determines the pro-protein (frame 1). The leading segment in 5'3' frame 1 is packed with hydrophobic residues (F, V, L, I, A) consistent with a signal peptide. No cleavage motifs were predicted so sequence "GWWKRVFKPVEKLGQRVRDAGIQGLEIAQQGANVLATARGGPPQQG" immediately after signal pro cleavage was used for structural predictions.

Sequence ID(s)	Predicted Class	Prediction Probability
TTCAAATAGACAAATTACCAAACTACAAAGCCAAAATGAATTCACAA/ TTGTTCTTATTGCCTTCTCCGGTCAAAGTG...	AMP	0.9999

i Probabilities > 0.5 = Predicted AMP

Figure 4.30 AMP prediction results for the cecropin-like peptide 1 using AMP Scanner v2. The nucleotide sequence was classified as an antimicrobial peptide (AMP) with a prediction probability of 0.9999, exceeding the classification threshold of 0.5.



Protein Type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.9953	0.0047

Name	Pos	Context	Score	Pred
Sequence	5	--MNFTK LF	0.057	.
Sequence	27	SEAGWWK RV	0.075	.
Sequence	28	EAGWWKR VF	0.231	.
Sequence	31	WWKRVFK PV	0.319	.
Sequence	35	VFKPVEK FG	0.064	.
Sequence	39	VEKFGQR VR	0.085	.
Sequence	41	KFGQVR DA	0.200	.
Sequence	62	NVLATAR GG	0.085	.

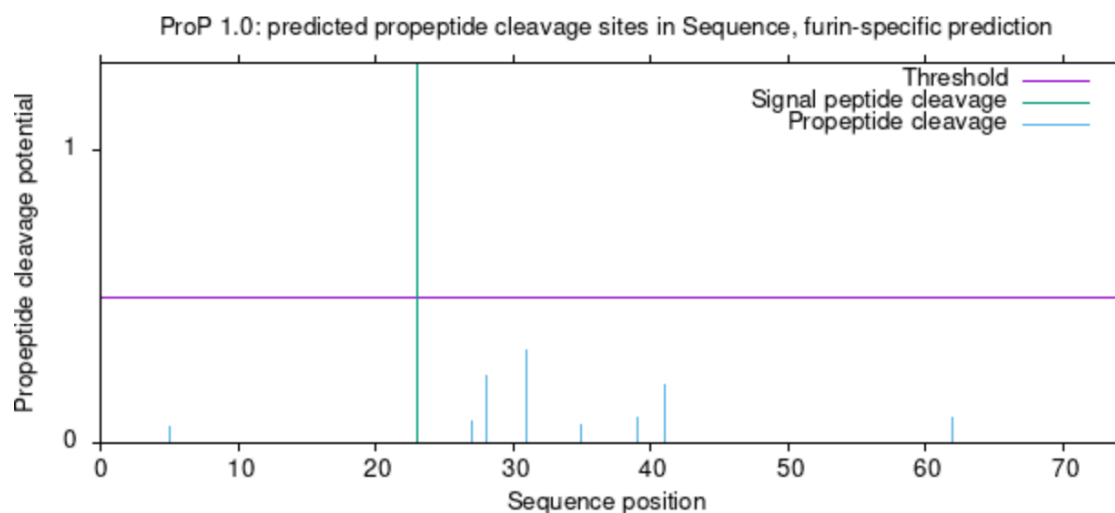


Figure 4.32 ProP (furin-specific mode) profile for cecropin-like peptide 1. Blue lines mark candidate convertase cleavages; the pink line is the default threshold; the green line (~23–24) marks the SignalP cut. No peaks rise above threshold, consistent with Table 13.

The mature cecropin-like peptide 1 was modelled from the sequence immediately downstream of the SignalP-5.0 cleavage (SEA↓GW at 22/23; model starts at residue 24) (Figure 4.33). The AlphaFold/ColabFold model converged to a single, continuous α -helix with a short low-confidence tail at the extreme C-terminus, exactly the architecture expected for cecropins, which are disulfide-free, amphipathic, α -helical AMPs (Chapman, 1998).

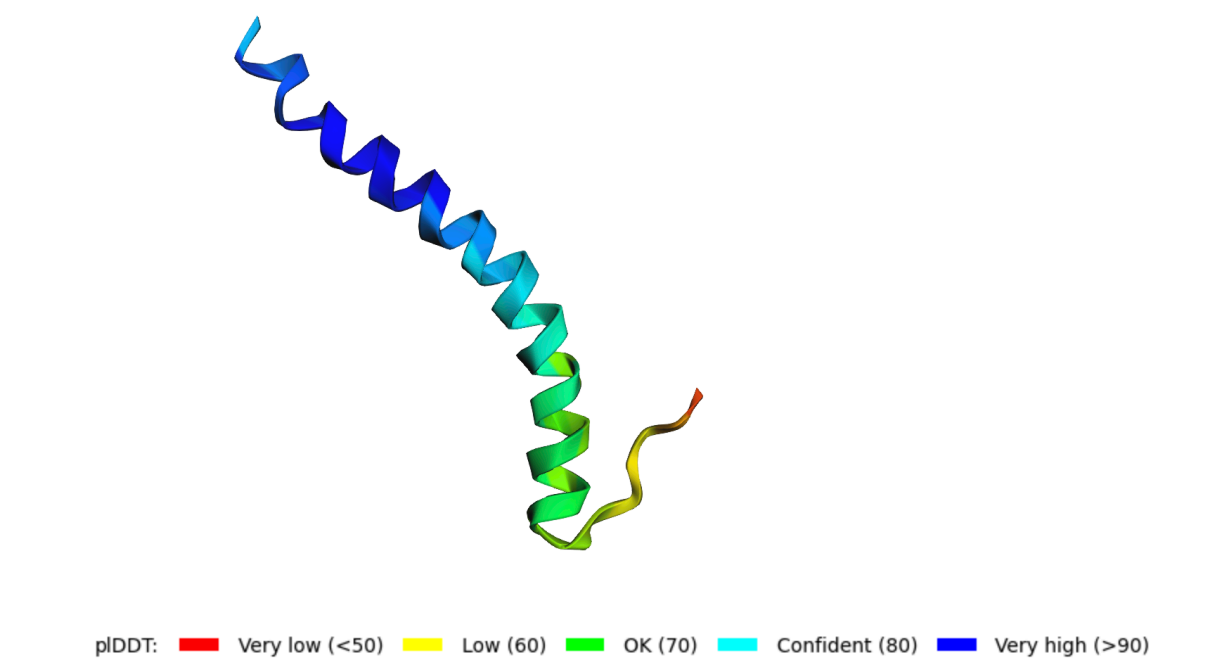


Figure 4.33 Predicted tertiary structure of Cecropin-like peptide 1 generated using AlphaFold. The model displays a characteristic amphipathic α -helical structure, typical of cecropin-family antimicrobial peptides.

4.35. Toll-Like Receptor 6 Screening

We next performed in-silico characterisation of a *Hermetia illucens* Toll-like receptor 6 (TLR6) (XP_037908134).

Expasy revealed the longest ORF (open reading frame) (Figure 4.34), and SignalP 5.0 classified the N-terminus as a Sec/SPI signal peptide (Figure 4.35; Table 4.15), with a predicted cleavage between positions 37-38 (AGA|QC); which was used as the predicted mature sequence for structural analysis (Figure 4.36).

KMLRSRVPSSSTH-TIQEIAFNLLKSGRG-NLTLDNWTLLGGRYVTRSCRRWIAFAAG-IRGVVWKSRLRIERNIRKNSQWLIRFNKAKIPSAVSI-IFCLV
L-KVCRVVKMSPPRSLELFPFSRLPSATAVLAVLAVIWTVVVRPAGAQCSWEYQPSSVKCHLRILESGQLNLQVAESADRLDIECSETLLFQSELPAEA
FARLPKLSLELQIESCRLLKLPNNAFKGLNSLRRLAVNTRNSIWGAGKSLEVFTSSNLGLRELQVLDLSENNLRQLPEGVWCPLGNLQILNLTHNRLR
SADSLGFGDLPCTGAGGSELQVLDVSHNELRISPDNWGISRLRRLNDLKLEFNNTIETSPDALSGLASLKIFNISYNHLETLPDTIFAGNKQLREIH
LQHNQIYIDLPGKIFHHLEELVLVDLSGNQLTSHNVDNATFSGLIRLVVLNLANALTRIGAKTFSELYFLQNLDLRNSIGHVEEGAFLPLYNLHTL
NLAENRLHTLDNKLFLNGLFILTKLTNNNLINIIESQAFRNCSDLKELDLSSNQLTEVPEATQDLAVLKTLDLGENQISGFGQNGSFRNLNQLTGLRL
IDNQIGNITIGMFWDLPRLSVLNLAKNRVQAIERGSFDKNTELEAIRLDRNLLTDINGVFATLASLLWLNLSENHLVWFYAFIPSKLKLWDIHGNY
TEALGNYYKLQEEIRVKTLDAASHNRITEIGPMSPVNTIELLFINNLIINTIHPNTFVDKTSLTRVDLYANMISKQLHLNLRVAPAPDEKMVPEFYLG
GNPFECDCSMEWLQRADSLTRSRQHPRIMDLANIECLMPHSRSAPIRALTSLSSSDFLCKYDTHCFALCHCCDFDADCCEMTCTPTNCSCYNDDRWGTN
IVDCGRQNTNQLPSKLPMDVDTLDLGNLVLFPQNQIFIGRKNLSRVYLNASNVTQNVQNTFAGLAALIEHLNNKLLQLTGSEFSGLSLKLEYL
HNNQLTSFIGNNTLADLSLRVLRIDNRLNPLVTQWQLHNLQKSSLTALAVGNMVSCHCKFLQELTEFVSSSSMVIQDAQDQIVCDVGAVKRELDLN
ASAGCNEQYAGNTAAIPNQDVYTGYPLLAAVLVLIVLIIQLLIVFVFRESVRMWLFAHYGVRVCSERSFDDAGKLYDAIILHSAKDYEFVCRTIAGE
LEHGRPPFRLCIQQRDLPPDASHLQIVEATRASKRMIVILTRNFLQTEWIRTEFRSAFHEALRGLTSKLVFIEETDVGPEAEVEVAELQPYLKSIPSN
RILTSDPYFEWELRYAIPIELSPRGNNYTI DHHERFKQVPSPGVIFRQAPPPPAYQDGETNYSSATTATPSRPARHPLNDIPMRPPSEHIYSSI
DSEYSGYDHENLSMIPSGAGAI CNPQHMKQLKHTAATTSGGHPSNWRPLNTAAVHASIHRSSGAQPQPQTPVAGSSSGSGTSTAPAPAGGANSSG
NTNSGQAFIV-RLARLTRPPTSHLILGTNGYERTANRKTSASAHHAMYO-QORAQPTN-KRSWLRF-LVGGCVCKSLEVNPNTRHNSL-HKETNSCDI
IVLTLFYFSITYTVSCKDVLVRTALMTQCLDISACK-YFMVKMP-N--GTGGSGHRGIDMRLGVNIDAI-TVEFGTFFPA-VKPLNDYDATKLQV-T
RYKST-DRVILQLPGRPIRPVLVDIHLGIRD-IHPKKCIYILSVHSSSLM-TLRSTALKGLCKLASPLNKSSYKRAQLLQQSRLTELNSKPHKAT-
PSSKLTADTATKASRPN-IIDQKTL-TGIF-TLIIIVIFQNETLLFR-TWLDYFLSPFLLLERGKNHFCVKHPINLRKIQFNGVTFFKLIACVSSTNE
L-FREPLLIINARIKPIPIHLFLI-IFSKNL-IINGKCRDNFLGRYAH--LNQSQRPRTL SATCGFYNNFNSYEATVLARTTHMNTYATR-WAIRT
KIKKNLHKFPPIHAKKKFR-MSGQYRALN-LLLLPVNLLS-FTPKLRAD-ITARSS-NVIFWILLLRIDK-FFWVGPERLKLWLLPLYIRISQKFP
IT-KENNELLLSSIT-KKKLTKKKIDVKI-SLTDs-K-EESDKTHFLVLLRFEV-IKKEDIIIEAGTLKISTEKFPDKTRNSFRQLNSVIFLWQ
KDFHVCVOLKSAFWAKRNSINLYVIFI-F-NSI-F-VG-ENFS-KS-NCIYSYDILCYVCIRLIGNKLFLLFVD-TELKEMYL-KSRFHKI-KNI

[illegible]

210

Table 4.15 SignalP-5.0 prediction probabilities for Toll-like Receptor 6. The model predicted the presence of a classical Sec/SPI signal peptide with a likelihood of 0.7098, while the probability for non-secretory classification was only 0.2902.

Protein Type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.7098	0.2902

Using InterPro/Phobius (v5.75-106.0) placed predicted residues 1-949 of the mature sequence as non-cytoplasmic (extracellular), comprising tandem Leucine Rich Repeat (LRRs) modules characteristic of TLR ectodomains, followed by a single transmembrane helix at 950-973 (TMHMM/Phobius) and a cytoplasmic tail (974-1323) containing a well-supported TIR domain (~992-1139 by PROSITE; 1017-1095 by Pfam). This architecture: LRR (leucine rich repeats) > ectodomain > single TM helix > cytosolic TIR, is the canonical TLR layout (Bell, et al., 2003). Together, these features identify it as a pattern-recognition receptor that binds ligands outside the cell and triggers intracellular immune signalling (Duan, Du, Xing, Wang, & Wang, 2022).

The long middle segment contains many tandem LRR motifs annotated by multiple databases (SMART/PFAM/InterPro), forms a curved, horseshoe-like scaffold. LRR surfaces are classic protein-ligand interfaces, and, in TLRs, they provide the binding platform for microbial patterns or endogenous ligands (Pålsson-McDermott & O'Neill, 2007) (Dolan, et al., 2007). A short hydrophobic helix near the C-terminus crosses the membrane once (single-pass). This places the LRR region outside the cell and the signalling domain inside. The intracellular tail contains a conserved TIR (~200 aa) domain annotated by InterPro/SMART. In both insects and mammals, TIR domains nucleate assembly of adaptor proteins (e.g., MyD88) and kinases that propagate signalling; in *Drosophila*, the canonical complex is MyD88-Tube-Pelle downstream of Toll, culminating in NF- κ B-like transcriptional responses (Lindsay & Wasserman, 2014).

Short low-complexity/disordered patches and predicted coiled-coil elements appear in the map. Disordered linkers often provide flexibility or regulatory sites, while coiled coils can mediate subunit interactions, features frequently observed in large receptors (Dyson & Wright, 2005) (Park W. , 2020) (Burkhard, Stetefeld, & Strelkov, 2001). These

annotations are consistent with a modular receptor whose ectodomain binds ligands and whose cytoplasmic tail recruits signalling adaptors.

Putting these tracks together: the protein is a Toll/TLR-type pattern-recognition receptor, LRR-rich ectodomain (recognition), single TM (membrane anchor), cytosolic TIR (signal initiation).

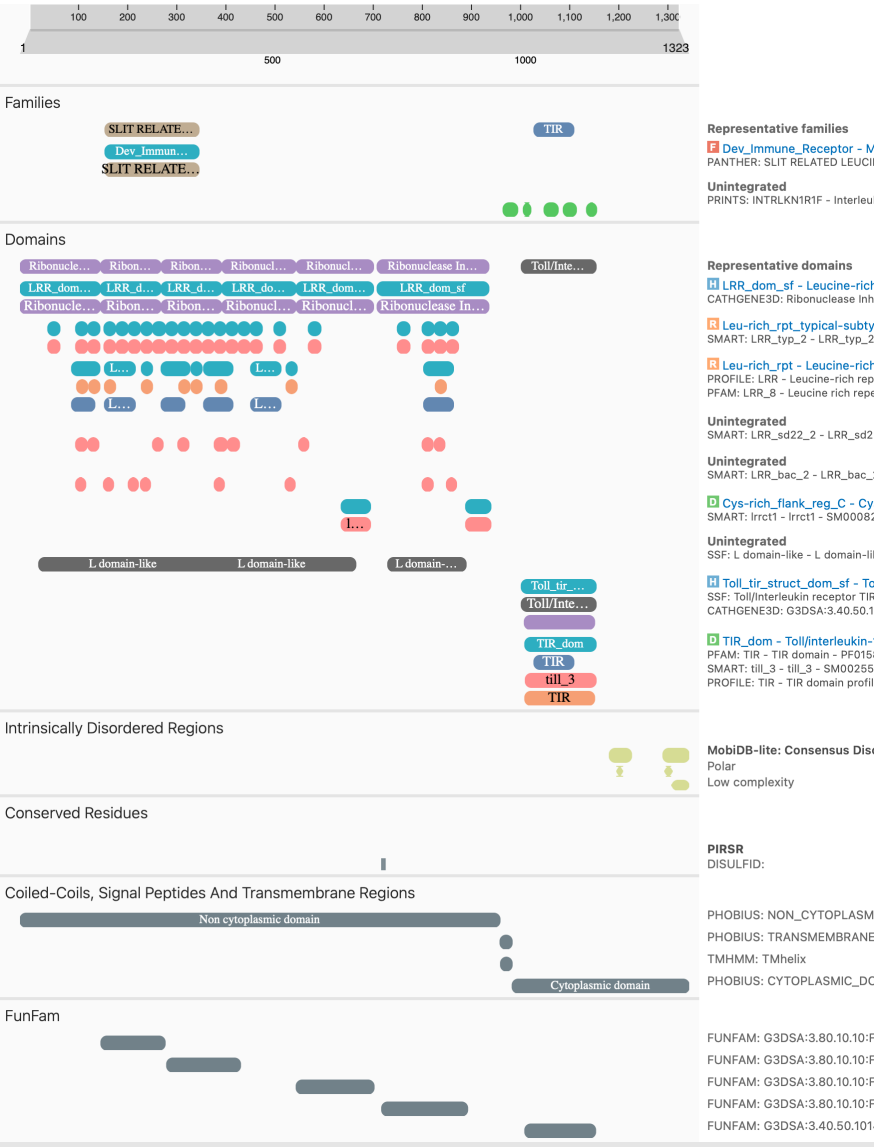


Figure 4.36 InterProScan of the mature sequence of Toll-like Receptor 6 shows multiple LRR units across the N-terminal ectodomain and a C-terminal TIR domain, separated by a single predicted transmembrane helix i.e., the canonical architecture of a Toll-like receptor. Sequence length predicted at 1323 amino acids.

4.36. Primers Design for downstream qPCR analysis of immune stimulated *Hermetia illucens*

Our candidate AMP and PRR sequences were collated from literature and public databases and successfully screened with validation tools before primer design (Table 4.16). From this process, seven targets progressed to full qPCR evaluation: three defensin-family AMPs (Defensin-A-like, Defensin-B-like, Defensin-like peptide 1), one Cecropin-like peptide 1, two Attacin-A-like paralogues (paralogue 1 and paralogue 2, designed to discriminate closely related transcripts), and Toll-like receptor 6 (TLR6). Several additional candidates were tested during development but were removed due to suboptimal specificity, inconsistent amplification, or poor efficiency during qPCR optimisation; only the seven primer sets listed in Table 15 satisfied all criteria and were carried forward to the immune-challenge screen.

Table 4.16 Forward/reverse primer sequences (5'>3'), sequence accessions used for design, annealing temperatures applied during optimisation, and the source of each reference sequence. Seven targets met all design and performance criteria (specific in-silico BLAST hits to the intended locus, acceptable secondary-structure/dimer scores, clean PCR QC, and satisfactory qPCR efficiency and melt behaviour); earlier candidates that failed design or bench tests were excluded.

Gene Name (In this study)	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Sequence ID	Annealing Temp (°C)	Sequence Source	Primer Design
Defensin-A-like	TCGTCCCATGGCAA TACAAT	TAGTGGAGCAGCATT ATCGGG	XM_038070404	57	(Candian, Meneguz, & Tedeschi, 2023)	(Candian, Meneguz, & Tedeschi, 2023)
Defensin-B-like	GGACGAAACGTGC AATGTGT	TGATCATCGCACCAG CCTC	XM_038071108.	57	(Vogel, Muller, Heckel, Gutzeit, & Vilcinskis, 2018)	this study
Defensin-like-peptide 1	GCTGTCTACACCTC AGCACA	TACAAACGGCTCGA CCATCG	KF805347.1	57	(Vogel, Muller, Heckel, Gutzeit, & Vilcinskis, 2018)	this study
Cecropin-like peptide 1	TTTTCGTTGTCTTCG CCGTT	ACTCTCTTCCACCAA CCAGC	XM_038063061.1	57	(Vogel, Muller, Heckel, Gutzeit, & Vilcinskis, 2018)	this study
Attacin-A-like (paralogue 1)	CAGGGCAGGACAT GGTCTTT	CCACCAAACGTTCTC GATGC	XM_038060438.1	58	(Vogel, Muller, Heckel, Gutzeit, &	this study

Attacin-A-like (paralogue 2)	ACGCTAGACTTTGG GAGGGT	CCAACTCCAGCATCT GGTCG	XM_038056655.1	58	Vilcinskas, 2018) (Vogel, Muller, Heckel, Gutzeit, & Vilcinskas, 2018) NCBI	this study
Toll-like Receptor 6 (TLR6)	GCAGGACTGGTTTC GACTCA	AACTGCGTATTCGTG GTGGT	XM_038052206.1	58		this study

4.37. Primer Optimisation: Determining optimal annealing temperature for efficient PCR/qPCR.

A temperature gradient rather than an assumed set-point was used to identify the most specific annealing conditions for each primer pair (Figure 4.37). TLR6 produced a clean, single 119 bp band across the mid-range of the gradient, with best specificity/intensity around ~58°C, and this primer set was taken forward to qPCR and generated the final dataset. TLR7 (183 bp) and MM1 (401 bp) also yielded discrete products at their expected sizes across the gradient and were successfully cloned (confirming primer specificity), but they didn't progress to the final stages of qPCR analysis due to downstream performance/throughput constraints. The RPL5 housekeeping primers amplified consistently across the gradient with a single product, supporting their use as the reference gene (Lu, et al., 2022).

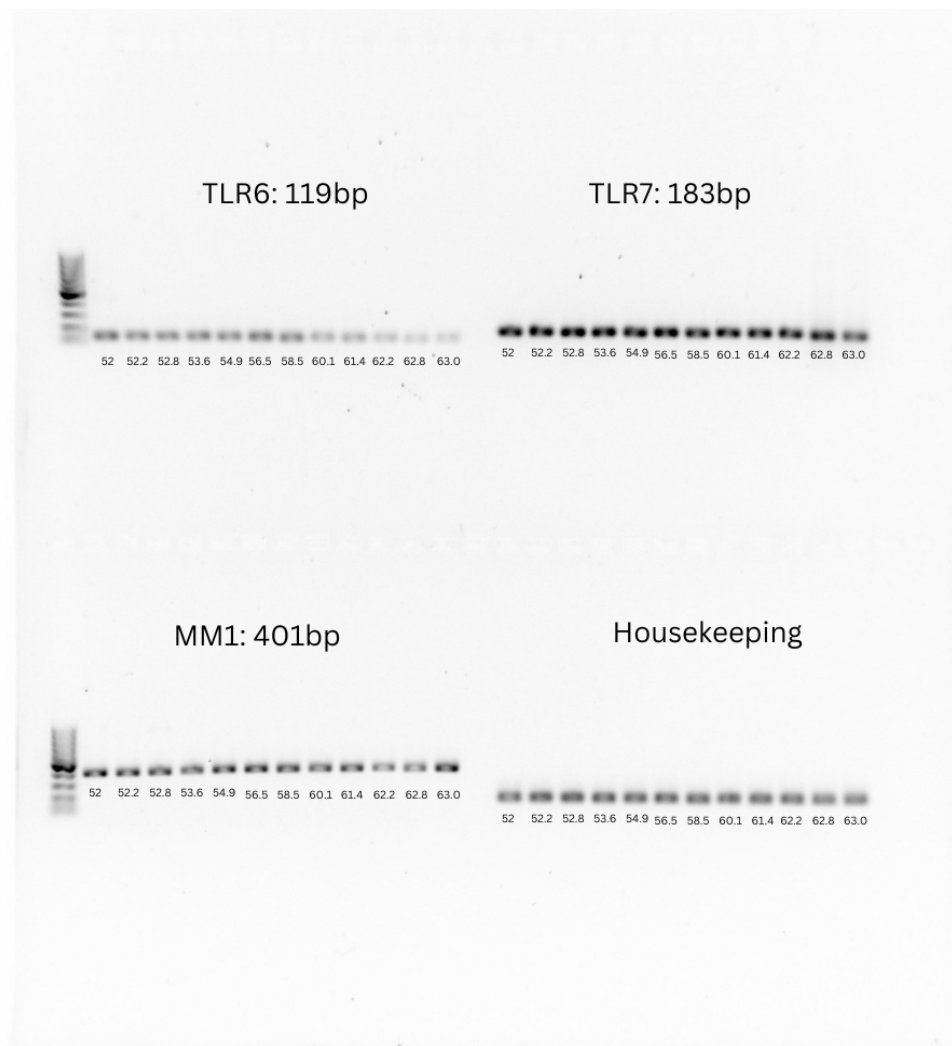


Figure 4.37 Gradient PCR (52–63 °C) was used to determine annealing temperatures for Toll-like Receptor 6; TLR6 (119 bp), Toll-like Receptor 7; TLR7 (183 bp), Macrophage Mannose Receptor; MMR/MM1 (401 bp), and the housekeeping gene RPL5. A PCR size ladder is shown in the leftmost lane of each panel; the prominent dark band corresponds to ~500 bp, used to verify product sizes (expected bands indicated above each panel). Reactions showing a single, discrete band at the expected size without primer-dimers were considered optimal and used to set qPCR annealing temperatures. Each primer also had a negative control (not shown here).

4.38. Determining Baseline mRNA abundance levels for Control, PBS and Injection Only

Larvae from control groups and sham injection groups (IO) stayed at baseline levels according to Defensin-B-like expression analysis (Figure 4.38). Suggesting that mechanical injection isn't causing any observable changes in gene expression. However, PBS groups see widespread increases, which could be attributed to the volume injected (10ul injected into 100mg larvae).

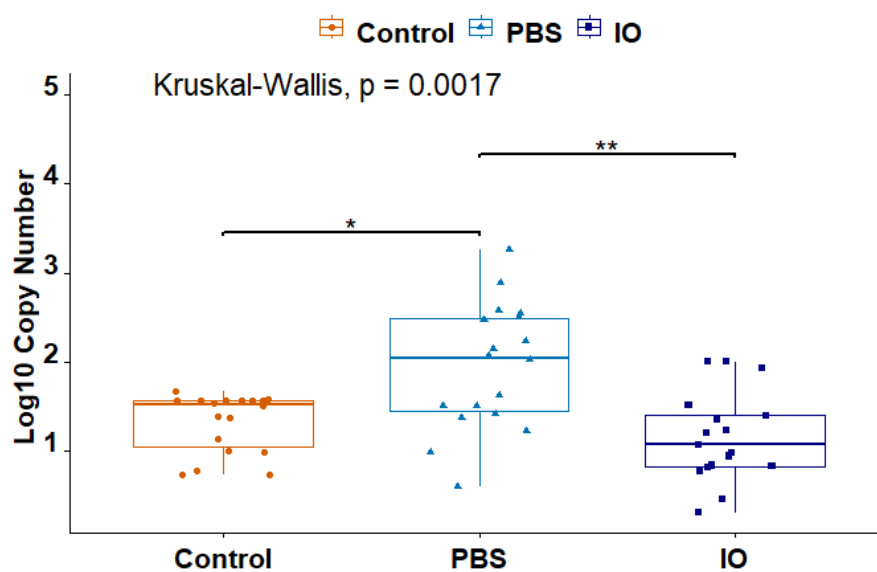


Figure 4.38 Determining baseline levels using Defensin-B-like as a reference point. Expression levels from Control (no treatment), 10 μ l of PBS and Injection Only (IO) (no volume, sham injection). Sham injection stays around baseline levels, whereas PBS/volume cause transcriptional changes.

4.39. Gene expression profile of Antimicrobial Peptide Defensins

The expression profiles of Defensin-A-like, Defensin-B-like and Defensin-like-peptide 1 exhibit distinct yet overlapping trends across treatment groups (Control, PBS, Poly I:C, and LPS), highlighting their role in the immune response of *H. illucens*.

Defensin-A-like demonstrates a significant difference between basal levels (Control) and PBS ($p < 0.0001$), showing measurable immune activation in response to injection-

stress and/or PBS (Figure 4.39). Both Poly (I:C) and LPS were found to be significantly different to PBS ($p < 0.0001$), indicating a significant change in mRNA abundance specific to pathogen-associated molecular patterns (PAMPs), and the ability to respond to both viral, and bacterial-like stimuli.

Defensin-B-like mRNA abundance exhibited significant changes between PBS and Control ($p > 0.0001$), but both treatment groups, Poly (I:C) and LPS, showed significant differences between PBS ($p > 0.01$ and $p > 0.001$, respectively) (Figure 4.40). While for Defensin-like-peptide 1 no measurable significant differences between baseline levels and PBS were observed however significant changes were identified between PBS and the treatment groups Poly (I:C) and LPS ($p > 0.0001$).

As previously discussed, Defensin-B-like and Defensin-like-peptide 1 share a high degree of similarity with 81.89% sequence identity, suggesting that they are closely related isoforms with potentially overlapping or complementary functions, further supported by very similar transcriptional responses to both Poly (I:C) and LPS (Figure 4.41). However, their non-identical sequences could indicate that there may be subtle differences in their antimicrobial properties or regulation. Notably, Defensin-B-like demonstrates a more robust, specific response at 2 μg , which could suggest that it operates in a concentration-dependent manner when stimulated by PAMPs, potentially reflecting functional divergence between the two isoforms.

The evolutionary divergence of these defensins aligns with the principle of gene duplication and diversification, enabling fine-tuning of the immune response. In the context of immune stimulation by bacterial lipopolysaccharides (LPS) and viral double-stranded RNA analogue poly(I:C), these defensins may exhibit distinct expression patterns or functional profiles.

While defensins are primarily recognised for their antibacterial activity, emerging research has identified their potential antiviral properties (Fu, et al., 2023). The data presented here demonstrate that *H. illucens* defensins exhibit increased transcriptional activity when exposed to LPS (a bacterial mimic) and Poly I:C (a viral mimic), reinforcing

their versatile role in the insect's innate immune defence. While the data are varied, there are also signs of concentration-dependent responses across some of the Poly (I:C) and LPS groups, suggesting that higher concentrations of these stimuli elicit stronger immune activation, highlighting sensitivity and adaptability of Defensins to varying level of pathogen-associated molecular patterns.

In line with the above, there is potential for the LPS preparations to contain contaminating PAMPs as previously published (MacKenzie, Roher, Boltaña, & Goetz, 2010), therefore, changes in response at high doses may reflect increasing doses of peptidoglycans that are driving the immune response.

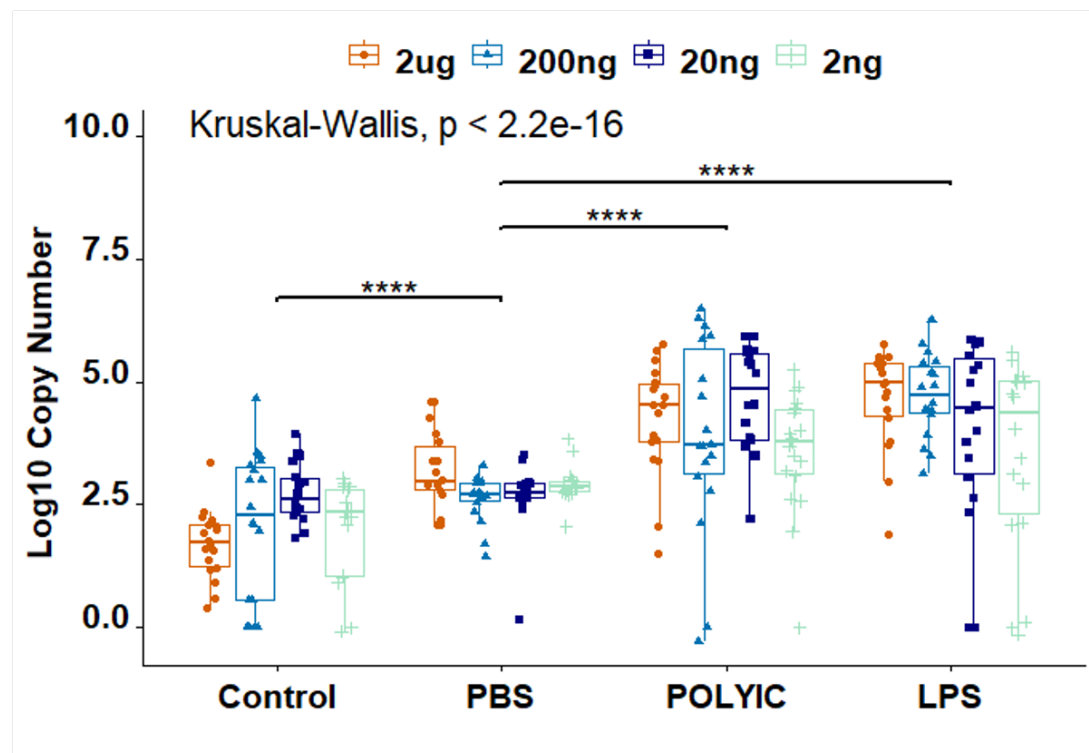


Figure 4.39 Expression of Defensin-A-like in *Hermetia illucens* Larvae Following PAMP Challenge

Box plot showing the log10 copy number of Defensin-A-like expression in *H. illucens* larvae at 24 hours post-injection with Control, PBS, Poly I:C and LPS (2μg, 200ng, 20ng, and 2ng) treatments, as determined by qPCR analysis.

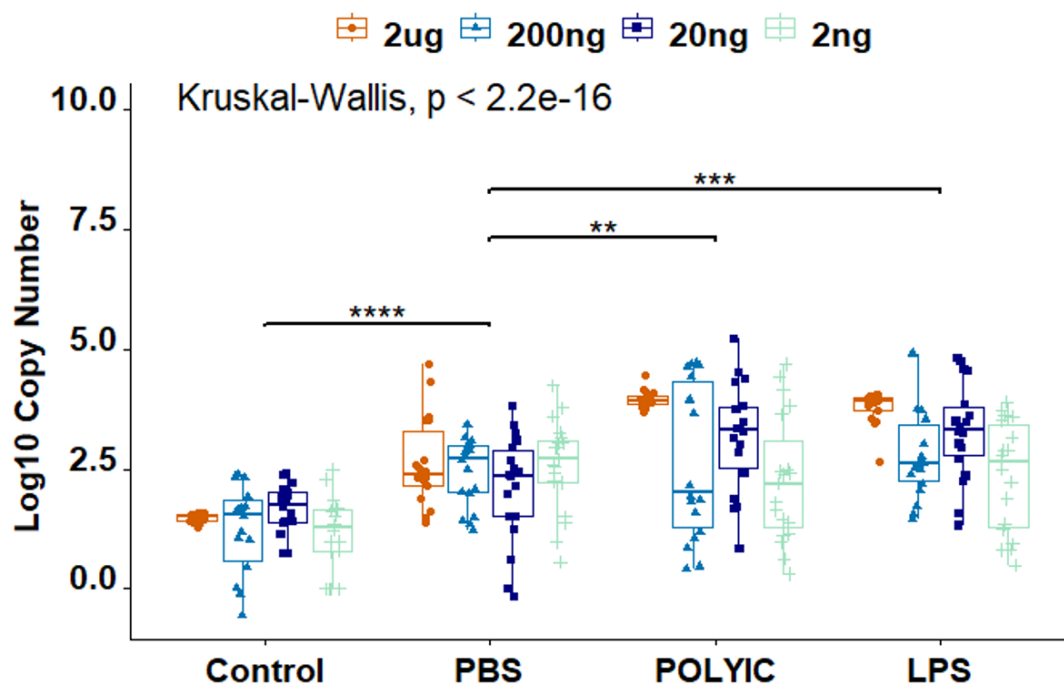


Figure 4.40 Expression of Defensin-B-like in *Hermetia illucens* Larvae Following PAMP Challenge

Box plot illustrating the log10 copy number of Defensin-B-like expression in *H. illucens* larvae at 24 hours post-injection with Control, PBS, Poly I:C and LPS (2 μ g, 200ng, 20ng, and 2ng) treatments, as measured by qPCR analysis.

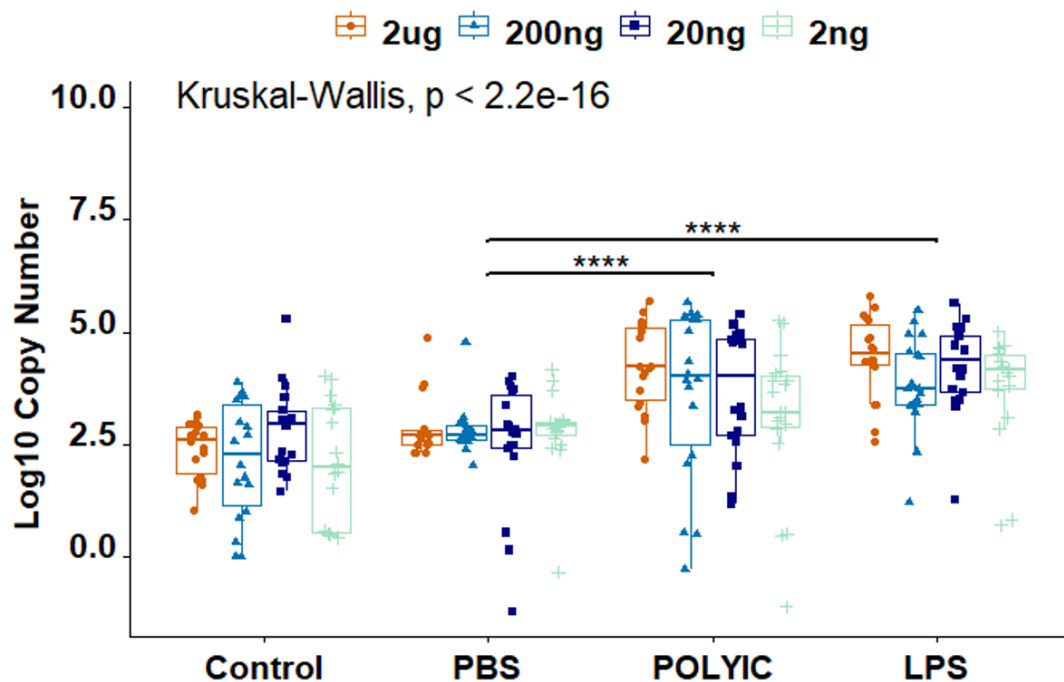


Figure 4.41 Expression of Defensin-like peptide 1 in *Hermetia illucens* Larvae Following PAMP Challenge

Box plot showing the log10 copy number of defensin-like peptide 1 expression in *H. illucens* larvae at 24 hours post-injection with Control, PBS, Poly I:C and LPS (2 μ g, 200ng, 20ng, and 2ng) treatments, as analyzed by qPCR.

4.40. Cecropin expression profile

The mRNA abundance of Cecropin-like peptide 1 shows increased transcription upon both Poly (I:C) and LPS stimuli (Figure 4.42). The Kruskal-Wallis test revealed a highly significant overall difference among treatment groups ($p = 2.2e-16$), confirming a robust transcriptional response to the experimental conditions. A significant difference can be seen between PBS ($p = 0.0001$), to baseline (Control), suggesting injection stress/PBS is causing transcriptional changes. However, a significant difference in mRNA abundance was determined for both Poly (I:C) ($p = 0.05$) and LPS ($p = 0.0001$) compared with PBS.

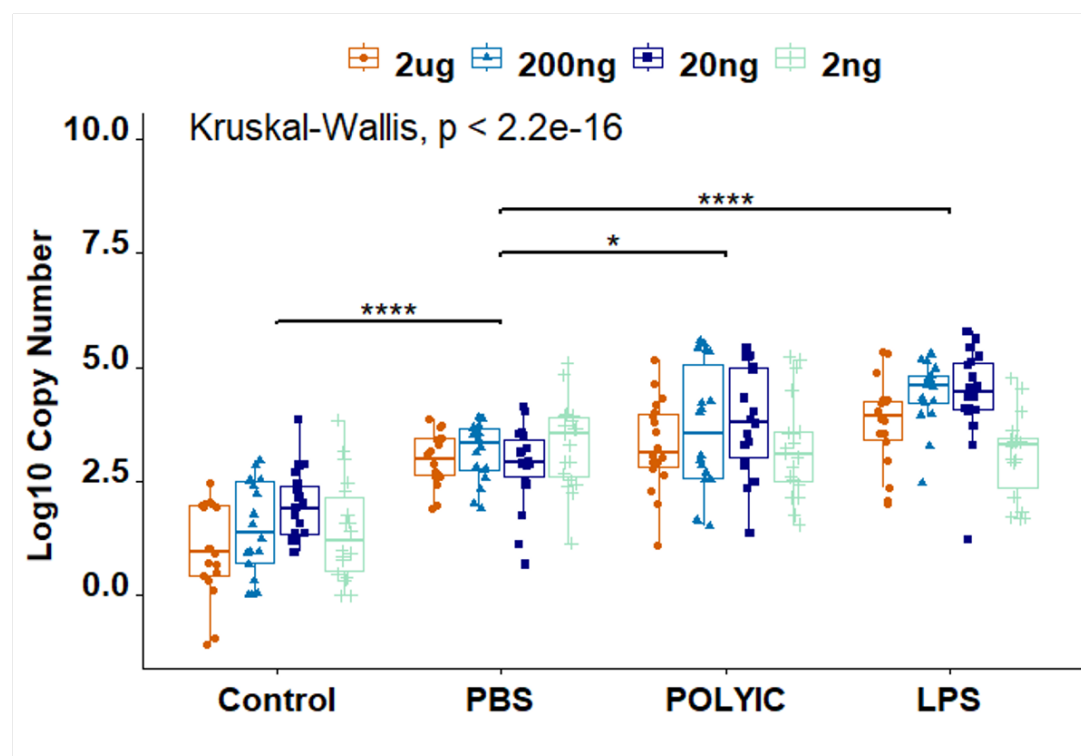


Figure 4.42 Expression of Cecropin-like peptide 1 in *Hermetia illucens* Larvae Following PAMP Challenge

Box plot showing the log₁₀ copy number of Cecropin 11A (Hill_SB_C698|cecropin) expression in *H. illucens* larvae at 24 hours post-injection with Control, PBS, Poly I:C (2μg, 200ng, 20ng, and 2ng), and LPS (2μg, 200ng, 20ng, and 2ng) treatments, as determined by qPCR analysis. Significant upregulation is observed in both Poly I:C and LPS-treated groups compared to Control and PBS, with Kruskal-Wallis analysis confirming overall significance ($p < 2.2e-16$).

4.41. Determining if Paralogues of Attacin-A-like behave differently after immune stimulation of LPS and Poly (I:C) in *Hermetia illucens*

The expression profile of the first paralogue: Attacin-A-like (Hill_BB_C16882|attacin) across the Control, PBS, Poly I:C, and LPS treatments showed significant variation, with significant differences between groups, confirmed by the Kruskal-Wallis analysis ($p < 2.2e-16$) (Figure 4.43). Expression levels remained low in the control group, indicating basal expression under unstimulated conditions. The PBS group exhibited slightly increased expression compared with baseline (Control) ($p < 0.0001$), perhaps attributed to mechanical injection stress/PBS. There appears to be a concentration-dependent response to LPS, showing a tapered response from 2 μ g-200 ng-20 ng-2 ng. No significance was found between Poly (I:C) and PBS groups; however, LPS was found to be statistically different to PBS ($P < 0.0001$), suggesting strong transcriptional changes to pathogen-associated molecular patterns (PAMPs).

The second Attacin-A-like paralogue (Figure 4.44) (Hill_BB_C10074|attacin-like) demonstrated a more consistent and wide-ranging response across replicates. This suggests a precise and robust transcriptional activation in response to both Poly (I:C) and LPS. This could reflect biological heterogeneity in immune activation. Again, PBS was seen to trigger transcriptional changes ($p > 0.0001$), however, strong significant differences were seen against both treatment groups and PBS ($p > 0.0001$), highlighting immune activation by both viral (Poly (I:C)) and bacterial (LPS) mimics.

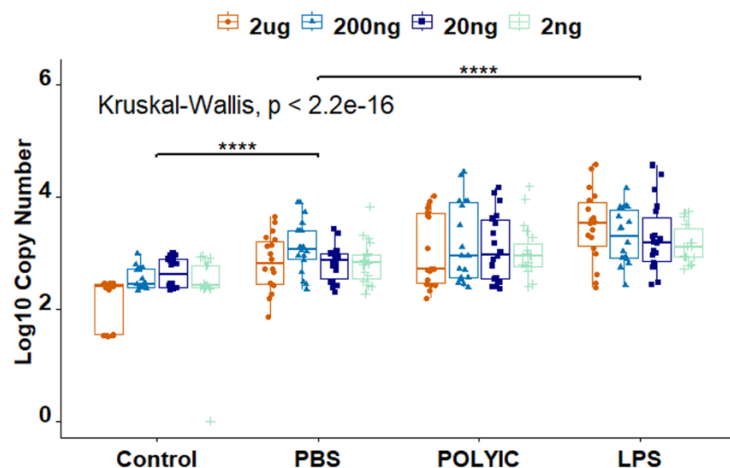


Figure 4.43 Expression of paralogue 1 Attacin-A-like in *Hermetia illucens* Larvae Following PAMP Challenge

Box plot showing the log10 copy number of Attacin-A-like (Hill_BB_C16882|attacin) expression in *H. illucens* larvae at 24 hours post-injection with Control, PBS, Poly I:C (2µg, 200ng, 20ng, and 2ng), and LPS (2µg, 200ng, 20ng, and 2ng) treatments, as determined by qPCR analysis. Poly I:C elicited the strongest upregulation, while LPS also significantly increased expression compared to Control and PBS groups ($p < 2.2e-16$).

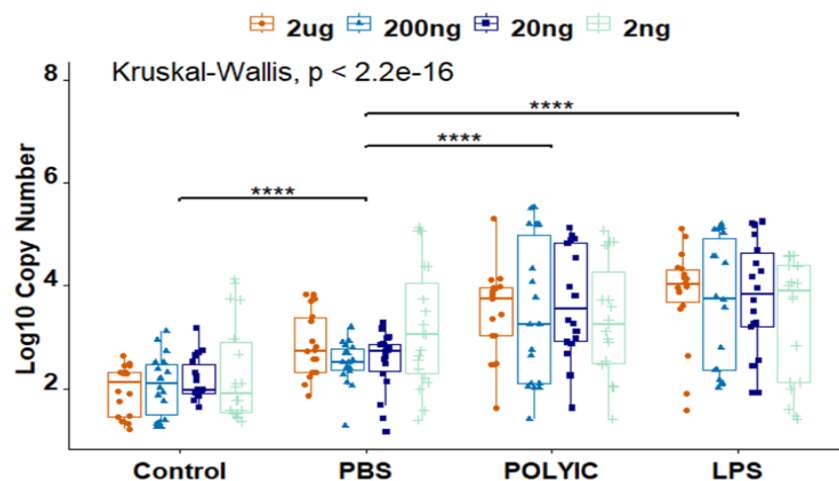


Figure 4.44 Expression of the second paralogue Attacin-A-like in *Hermetia illucens* Larvae Following PAMP Challenge

Box plot illustrating the log10 copy number of Attacin 13A (Hill_BB_C10074|attacin-like) expression in *H. illucens* larvae at 24 hours post-injection with Control, PBS, Poly I:C (2µg, 20 ng, 20ng, and 2ng), and LPS (2µg, 200ng, 20ng, and 2ng) treatments, as analyzed by qPCR. LPS elicited slightly higher median expression compared to Poly I:C, particularly at lower concentrations, with significant upregulation observed across all PAMP treatments ($p < 2.2e-16$).

4.42. Pathogen Recognition Receptor (PRR) Toll-Like Receptor-6 (TLR6)

Under basal, non-stimulated conditions (Control), TLR6 showed low expression, consistent with its expected role in pathogen detection rather than constitutive activity (Figure 4.45). The PBS group exhibited a significant difference from Control ($p < 0.0001$), likely attributable to mechanical stress caused by injection or PBS solution. However, a strong statistical difference was seen between both treatment groups and PBS ($p < 0.0001$), suggesting strong transcriptional activation due to the presence of PAMPs Poly (I:C) and LPS.

On a $\log_{10}$ (copy number) scale, TLR6 expression remained statistically stable across 2 ng, 20 ng, and 200 ng for both Poly(I:C) and LPS (Poly(I:C): 1.26 ± 0.14 , 1.28 ± 0.12 , 1.22 ± 0.12 ; LPS: 1.24 ± 0.13 , 1.33 ± 0.18 , 1.27 ± 0.13), with no significant differences among these concentrations (Holm-adjusted $p \geq 0.27$). In contrast, 2 μg , reached a mean of 1.64 ± 0.07 for Poly (I:C) and 1.58 ± 0.08 for LPS, potentially indicating a threshold concentration, or, as discussed before, contamination of peptidoglycans within PAMPs (MacKenzie, Roher, Boltaña, & Goetz, 2010).

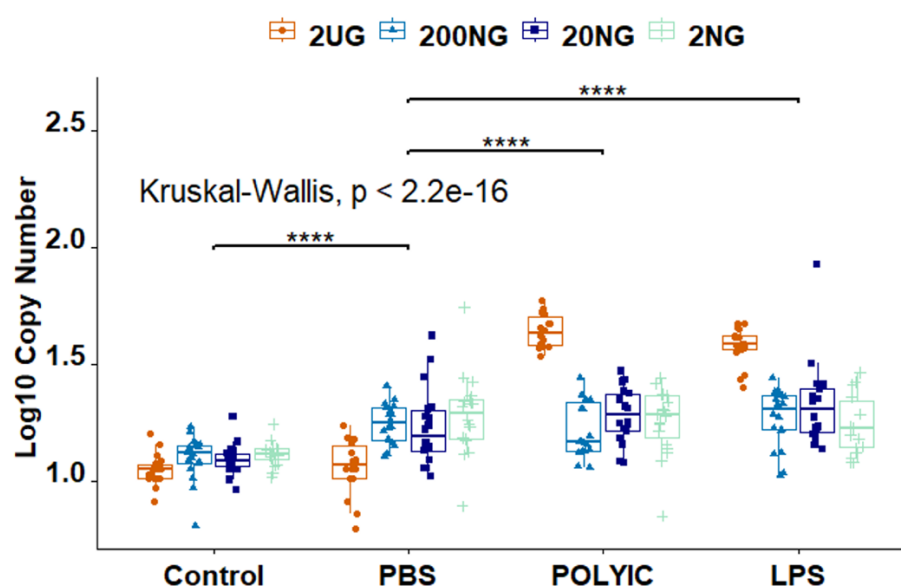


Figure 4.45 TLR6 Expression Profiles Across Treatments in *Hermetia illucens*. The boxplot illustrates $\log_{10}$ -transformed TLR6 copy numbers across four treatments: Control, PBS, Poly I:C, and LPS, at four concentrations (2 μg , 200ng, 20ng, and 2ng). Statistical analysis using the Kruskal-Wallis test revealed a highly significant difference among

treatment groups ($p < 2.2e-16$). Asterisks indicate significant pairwise comparisons ($p < 0.0001$). TLR6 expression demonstrates distinct upregulation patterns in response to pathogen-associated molecular patterns (PAMPs).

4.43. qPCR results by PAMP concentration

To visualise dose effects closer, expression data was re-plotted by individual PAMP concentration (2 μ g, 200 ng, 20 ng, 2 ng) across the four treatment groups (Control, PBS, Poly I:C, LPS) (Figure 4.46-4.49). Cecropin-like peptide 1 was significantly modulated by both LPS and Poly(I:C) in a dose-responsive manner. In PBS-treated samples, median log₁₀ (copy number) values ranged from 2.99-3.55, remaining relatively stable across each concentration.

For LPS, expression was significantly elevated at all tested concentrations, with the strongest induction at 20 ng and 200 ng (median log₁₀ values: 4.45 and 4.59, respectively). Expression remained high but slightly lower at 2 μ g (median: 3.95) and was also increased at 2 ng (median: 3.31) displaying a non-linear dose-response rather than a simple monotonic increase. Poly (I:C) showed a broadly similar trend with statistically significant induction across all concentrations. Together, these data show that cecropin-like peptide 1 is robustly inducible by both bacterial (LPS) and viral mimic (poly(I:C)).

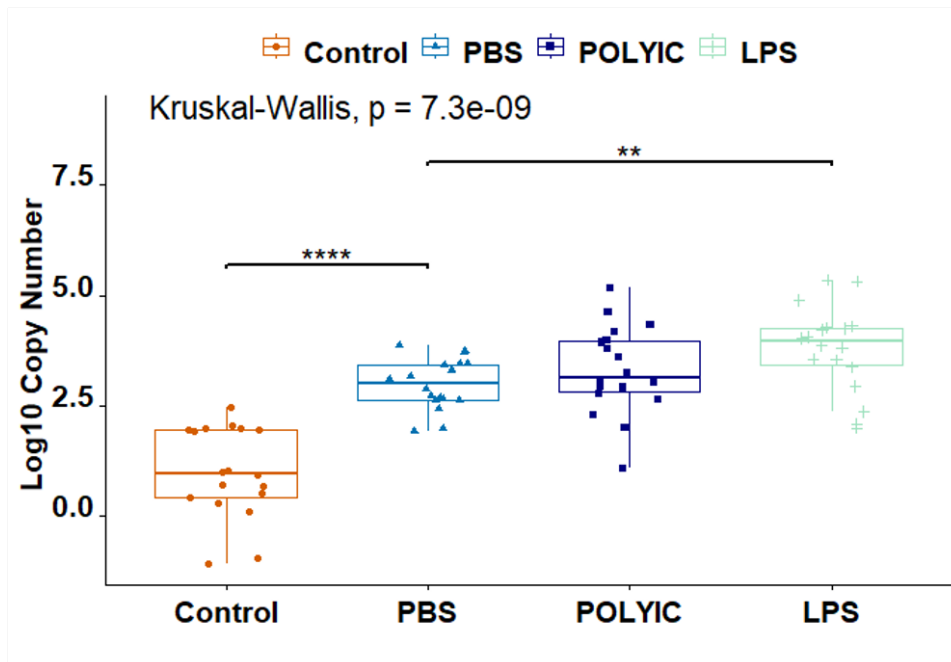


Figure 4.46 Cecropin-like peptide 1 mRNA abundance at 2µg Across Treatments in *Hermetia illucens*. The boxplot illustrates the log₁₀-transformed copy numbers of Cecropin-like peptide 1 across four treatments: Control, PBS, Poly I:C, and LPS at a concentration of 2µg. Statistical analysis via the Kruskal-Wallis test revealed a highly significant overall difference among treatment groups ($p = 7.3e-09$). Asterisks indicate significant pairwise comparisons ($p < 0.0001$, $p < 0.01$). Statistical significance was found between control and PBS groups and PBS and LPS groups.

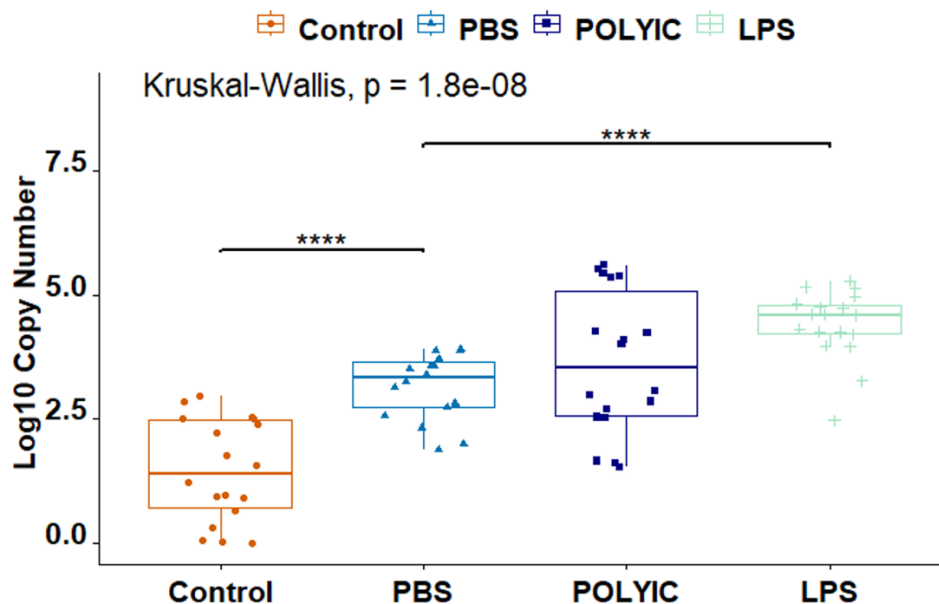


Figure 4.47 Cecropin-like peptide 1 mRNA abundance at 200ng Across Treatments in *Hermetia illucens*. The boxplot displays the log₁₀-transformed copy numbers of Cecropin-like peptide 1 across the treatment groups: Control, PBS, Poly I:C, and LPS at a concentration of 200ng. A Kruskal-Wallis test confirmed a highly significant overall difference among the treatments ($p = 1.8e-08$). Pairwise comparisons demonstrate significant upregulation of Cecropin-like peptide 1 in the Poly I:C and LPS groups compared to Control ($p < 0.0001$). Statistical significance was found between control and PBS groups and PBS and LPS groups.

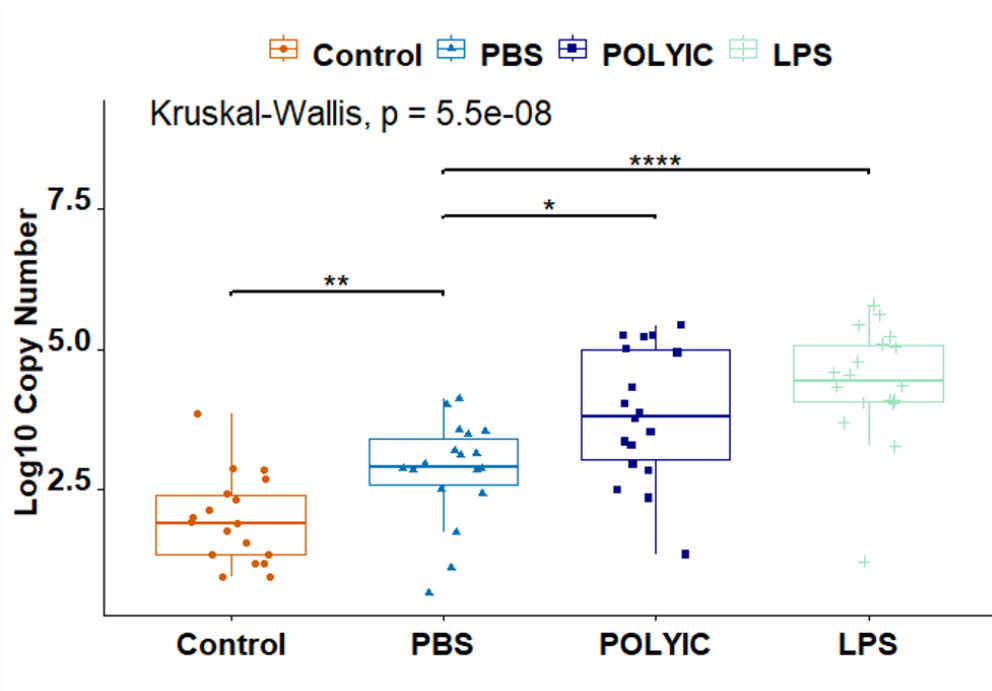


Figure 4.48 Cecropin-like peptide 1 mRNA abundance at 20ng Across Treatments in *Hermetia illucens*. The boxplot shows the log10-transformed copy numbers across the treatment groups: Control, PBS, Poly I:C, and LPS at a concentration of 20ng. The Kruskal-Wallis test indicated a highly significant difference among treatments ($p = 5.5e-08$). Pairwise comparisons reveal significant upregulation of Cecropin-like peptide in the Poly I:C and LPS groups compared to Control ($p < 0.0001$) and PBS ($p < 0.05$ for Poly I:C vs. PBS). Statistical analyses revealed significant differences between the Control and PBS groups ($**p < 0.01$), as well as between PBS and Poly (I:C) ($*p < 0.05$) and PBS and LPS ($****p < 0.0001$), highlighting a role in both viral and bacterial stimuli.

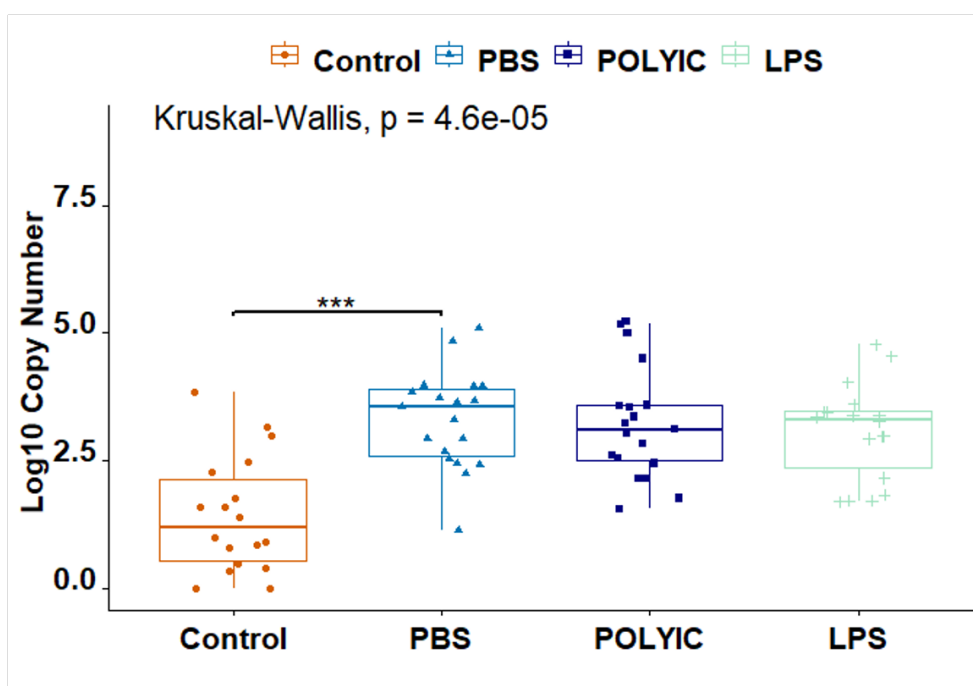


Figure 4.49 Cecropin-like peptide 1 mRNA abundance at 2ng Across Treatments in *Hermetia illucens*. The boxplot depicts the log10-transformed copy numbers across the treatment groups: Control, PBS, Poly I:C, and LPS at

a concentration of 2ng. The Kruskal-Wallis test revealed significant differences among the groups ($p = 4.6e-05$). Pairwise comparisons highlight significant upregulation in the PBS group compared to Control ($p < 0.001$).

4.44. Integrated analysis of immune gene expression

To complement the preceding gene-specific analyses based on $\log_{10}$ -transformed qPCR copy number, additional analyses were performed using absolute qPCR copy number, fold-change, linear modelling and dose-response correlation analyses (Figures 4.50-4.53; Tables 4.17-4.19). These analyses were undertaken to quantify the magnitude of transcriptional induction, assess dose-dependent responses across increasing PAMP concentrations and determine whether treatment effects remained significant when dose was included as a covariate.

Absolute qPCR copy number confirmed the transcriptional patterns observed in the preceding analyses, with Poly(I:C) and LPS consistently inducing greater expression of antimicrobial peptide genes than either untreated Control or PBS-treated larvae (Figures 4.50-4.53). Although the magnitude of induction varied between genes, antimicrobial peptides generally exhibited substantially larger increases in transcript abundance than Toll-like receptor 6, indicating a stronger downstream effector response following immune stimulation.

Fold-change analysis further quantified these responses by comparing stimulated groups with both untreated Control and PBS-treated larvae (Table 4.17). The greatest induction was observed for Cecropin-like peptide 1, Defensin A-like, Defensin B-like and Attacin A-like paralogue 2, with fold changes frequently exceeding two orders of magnitude at intermediate and higher PAMP concentrations. In contrast, Toll-like receptor 6 displayed comparatively modest fold changes, remaining below five-fold across all treatment comparisons.

Linear modelling demonstrated that treatment significantly influenced expression of every target gene (all treatment terms $p < 0.001$; Table 4.18), confirming that immune stimulation was the primary driver of transcriptional variation. Significant effects of

PAMP concentration were detected only for Defensin B-like and Toll-like receptor 6, while a significant treatment $\times$ dose interaction was identified exclusively for Toll-like receptor 6, indicating that receptor expression varied according to both treatment type and PAMP concentration. Model R^2 values ranged from 0.206 to 0.509, demonstrating that the fitted models explained approximately 21–51% of the observed variation in gene expression.

Spearman correlation analyses similarly showed that dose-dependent relationships were not universal across the immune-associated genes examined (Table 4.19). Following Holm correction, significant positive correlations between increasing PAMP concentration and transcript abundance were detected for Defensin B-like following Poly(I:C) ($p = 0.373$, adjusted $p = 0.030$) and LPS ($p = 0.432$, adjusted $p = 0.004$), together with Toll-like receptor 6 following PBS ($p = 0.476$, adjusted $p < 0.001$), Poly(I:C) ($p = 0.521$, adjusted $p < 0.001$) and LPS ($p = 0.574$, adjusted $p < 0.001$). No significant dose-response relationships remained following multiple-testing correction for the remaining antimicrobial peptide genes.

Although these analyses quantified treatment- and dose-dependent responses for individual immune-associated genes, they do not capture the overall relationships among genes across individual samples. Therefore, multivariate analyses were performed using Z-score normalised qPCR expression profiles to investigate global transcriptional patterns, identify similarities among samples and determine whether clustering was associated with treatment, stimulant concentration or biological replicate (Figures 4.54–4.56; Table 4.20).

Hierarchical clustering of all individual samples identified four principal transcriptional clusters based on similarities in overall immune gene expression profiles (Figure 4.54). While samples from all treatment groups were represented throughout the dendrogram, Poly(I:C)- and LPS-treated larvae were generally associated with clusters displaying greater antimicrobial peptide expression, whereas untreated Control and PBS-treated larvae more frequently exhibited lower overall transcriptional activity. Biological replicates were distributed throughout the dendrogram rather than forming

replicate-specific groupings, suggesting that the observed clustering reflected biological responses to immune stimulation rather than batch effects.

Principal component analysis similarly demonstrated that treatment accounted for much of the global transcriptional variation observed across the dataset (Figure 4.55). Principal component 1 explained 55.0% of the total variance, while principal component 2 explained a further 15.0%, together accounting for 70.0% of the overall variation in immune gene expression. Samples showed partial separation according to treatment, primarily along PC1, whereas differences associated with increasing stimulant concentration were more apparent along PC2. Nevertheless, overlap between treatment groups remained, reflecting the biological variability observed among individual larvae despite clear overall treatment-associated transcriptional responses.

Mean Z-score expression profiles of each hierarchical cluster further highlighted distinct transcriptional signatures (Figure 4.56). Cluster 1 was characterised by relatively elevated Defensin B-like expression, representing a Defensin B-like-enriched response profile. Cluster 2 displayed broadly increased expression across multiple antimicrobial peptide genes, including Attacin A-like paralogue 1, Attacin A-like paralogue 2, Cecropin-like peptide 1, Defensin A-like and Defensin-like peptide 1, consistent with widespread antimicrobial peptide activation following immune stimulation. Cluster 3 exhibited comparatively low expression across all immune-associated genes, representing baseline or weakly induced transcriptional profiles. In contrast, Cluster 4 showed relatively greater Toll-like receptor 6 expression together with comparatively lower antimicrobial peptide expression, indicating a Toll-like receptor 6-enriched profile associated with stronger immune stimulation.

Statistical analysis confirmed that treatment was the primary determinant of the multivariate transcriptional patterns observed (Table 4.20). Treatment significantly influenced both PC1 ($H = 147.22$, $p < 0.001$) and PC2 ($H = 78.13$, $p < 0.001$), whereas biological replicate showed no significant association with either principal component. Stimulant concentration significantly influenced PC2 ($H = 49.55$, $p < 0.001$) but not PC1, while Spearman correlation analysis similarly identified a significant positive

relationship between stimulant concentration and PC2 ($p = 0.303$, $p < 0.001$), with no corresponding association for PC1. Consistent with these findings, hierarchical cluster membership was significantly associated with both treatment ($\chi^2 = 62.56$, $p < 0.001$) and stimulant concentration ($\chi^2 = 74.83$, $p < 0.001$), whereas no significant association was detected with biological replicate ($\chi^2 = 47.24$, $p = 0.624$). Together, these findings demonstrate that treatment exerted the strongest influence on global immune transcriptional profiles, with stimulant concentration contributing to secondary variation, while biological replicate had minimal impact on sample grouping.

Collectively, the integrated analyses demonstrate that immune treatment was the dominant determinant of transcriptional activation across both individual immune-associated genes and global transcriptional profiles. Absolute copy number, fold-change, linear modelling and correlation analyses consistently identified robust treatment effects, whereas statistically significant dose-dependent regulation was largely restricted to Defensin B-like and Toll-like receptor 6. The multivariate analyses further demonstrated that treatment explained the greatest proportion of variation in overall immune gene expression and that biological replicates did not introduce systematic clustering. Together, these complementary approaches reinforce the conclusion that treatment identity exerts a substantially greater influence on immune transcriptional responses than increasing PAMP concentration, while confirming the reproducibility of the observed transcriptional patterns across biological replicates.

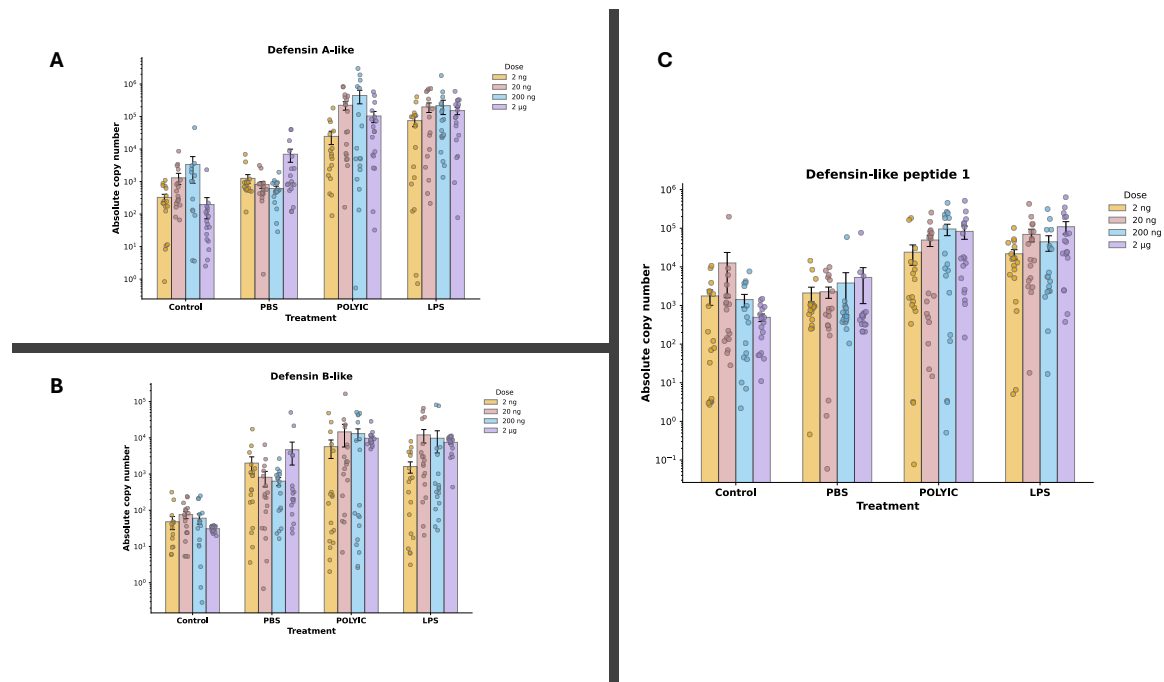


Figure 4.50 Absolute qPCR copy number of Defensin A-like (A), Defensin B-like (B) and Defensin-like peptide 1 (C) following immune stimulation with PBS, Poly(I:C) and LPS at four PAMP concentrations (2 ng, 20 ng, 200 ng and 2 µg). Bars represent the mean $\pm$ SEM and points represent individual biological replicates.

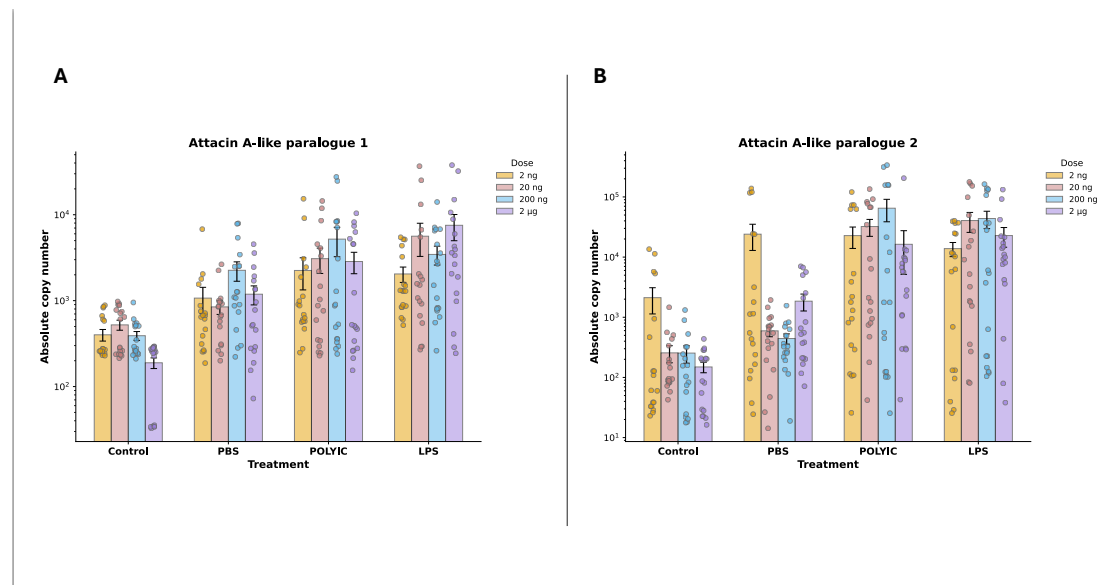


Figure 4.51 Absolute qPCR copy number of Attacin A-like paralogue 1 (A) and Attacin A-like paralogue 2 (B) following immune stimulation with PBS, Poly(I:C) and LPS at four PAMP concentrations (2 ng, 20 ng, 200 ng and 2 µg). Bars represent the mean $\pm$ SEM and points represent individual biological replicates.

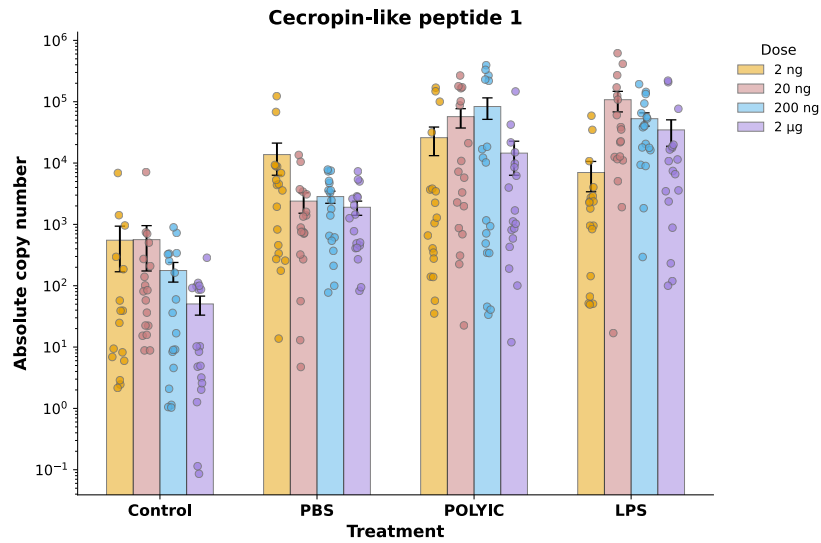


Figure 4.52 Absolute qPCR copy number of Cecropin-like peptide 1 following immune stimulation with PBS, Poly(I:C) and LPS at four PAMP concentrations (2 ng, 20 ng, 200 ng and 2 µg). Bars represent the mean ± SEM and points represent individual biological replicates.

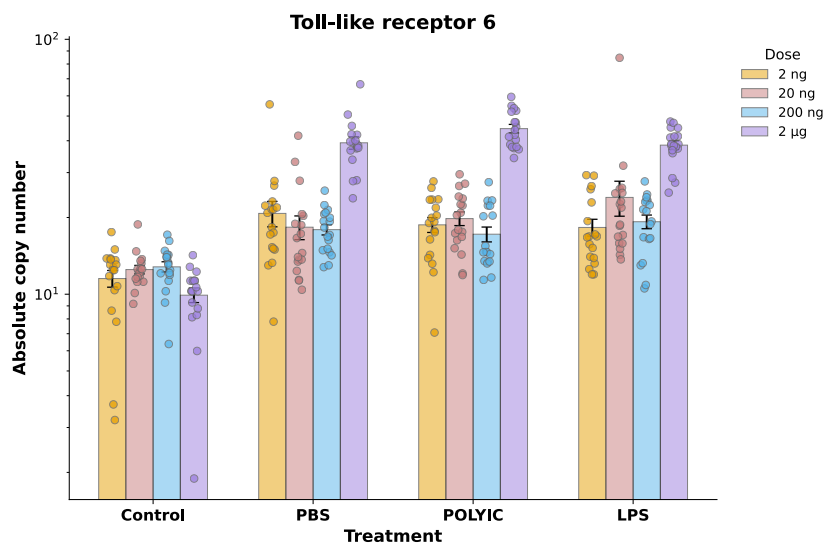


Figure 4.53 Absolute qPCR copy number of Toll-like receptor 6 (TLR6) following immune stimulation with PBS, Poly(I:C) and LPS at four PAMP concentrations (2 ng, 20 ng, 200 ng and 2 µg). Bars represent the mean ± SEM and points represent individual biological replicates.

Table 4.17 Fold-change in absolute qPCR copy number following immune stimulation with Poly(I:C) and LPS relative to untreated Control and PBS-injected groups across four PAMP concentrations (2 ng, 20 ng, 200 ng and 2 µg). Fold-change values were calculated using the mean absolute copy number for each treatment group divided by the corresponding mean value of the reference (Control or PBS) group at the same PAMP concentration. Values greater than 1 indicate increased expression relative to the reference group, whereas values less than 1 indicate reduced expression.

Gene	Dose	Poly(I:C) vs Control	LPS vs Control	Poly(I:C) vs PBS	LPS vs PBS
Attacin A-like paralogue 1	2 ng	5.61	5.11	2.10	1.91
	20 ng	5.90	10.75	3.64	6.64
	200 ng	13.28	8.83	2.30	1.53
	2 µg	15.12	39.83	2.40	6.33
Attacin A-like paralogue 2	2 ng	10.75	6.53	0.95	0.58
	20 ng	125.89	158.26	54.21	68.15
	200 ng	255.84	172.46	146.53	98.77
	2 µg	109.29	152.71	8.82	12.32
Cecropin-like peptide 1	2 ng	46.93	12.69	1.89	0.51
	20 ng	101.21	191.18	23.84	45.03
	200 ng	471.88	298.83	29.39	18.61
	2 µg	287.80	687.89	7.63	18.25
Defensin A-like	2 ng	75.33	229.46	19.61	59.74
	20 ng	170.36	151.94	274.66	244.97
	200 ng	131.86	64.13	723.98	352.12
	2 µg	528.79	776.98	14.99	22.02
Defensin B-like	2 ng	118.26	33.43	2.85	0.80
	20 ng	192.28	158.59	17.95	14.81
	200 ng	216.20	161.13	20.53	15.30
	2 µg	314.05	242.03	2.07	1.60
Defensin-like peptide 1	2 ng	13.58	12.35	11.37	10.34
	20 ng	3.93	5.50	21.82	30.57
	200 ng	67.20	31.30	25.05	11.66
	2 µg	168.21	221.75	15.57	20.53
Toll-like receptor 6	2 ng	1.62	1.59	0.90	0.88
	20 ng	1.59	1.92	1.08	1.31
	200 ng	1.34	1.50	0.96	1.08
	2 µg	4.50	3.87	1.14	0.98

Table 4.18 Summary of linear model analyses examining the effects of treatment, PAMP dose and treatment × dose interaction on absolute qPCR copy number for each immune-associated target gene. Linear models were fitted using treatment and log₁₀-transformed PAMP dose as explanatory variables. Reported p-values indicate the significance of each model term, while the coefficient of determination (R²) describes the proportion of variance in gene expression explained by the fitted model.

Gene	Treatment <i>p</i>	Dose <i>p</i>	Treatment × Dose <i>p</i>	Model R ²
Attacin A-like paralogue 1	<0.001	0.316	0.803	0.210
Attacin A-like paralogue 2	<0.001	0.799	0.268	0.305
Cecropin-like peptide 1	<0.001	0.506	0.416	0.419
Defensin A-like	<0.001	0.106	0.074	0.206
Defensin B-like	<0.001	<0.001	0.358	0.304
Defensin-like peptide 1	<0.001	0.070	0.260	0.250
Toll-like receptor 6	<0.001	<0.001	<0.001	0.509

Table 4.19 Summary of Spearman rank correlation analyses assessing dose-response relationships between PAMP concentration and absolute qPCR copy number for each immune-associated target gene. Correlation coefficients (*ρ*) and Holm-adjusted p-values are shown for each treatment group. Significant positive correlations indicate increased gene expression with increasing PAMP concentration.

Gene	Control (<i>ρ</i> , adj. <i>p</i>)	PBS (<i>ρ</i> , adj. <i>p</i>)	Poly(I:C) (<i>ρ</i> , adj. <i>p</i>)	LPS (<i>ρ</i> , adj. <i>p</i>)
Attacin A-like paralogue 1	-0.238, 0.928	0.117, 1.000	-0.053, 1.000	0.201, 1.000
Attacin A-like paralogue 2	-0.103, 1.000	-0.055, 1.000	0.057, 1.000	0.094, 1.000
Cecropin-like peptide 1	-0.132, 1.000	-0.130, 1.000	0.025, 1.000	0.223, 1.000
Defensin A-like	-0.242, 0.901	0.071, 1.000	0.142, 1.000	0.221, 1.000
Defensin B-like	0.039, 1.000	-0.008, 1.000	0.373, 0.030	0.432, 0.004
Defensin-like peptide 1	-0.017, 1.000	-0.180, 1.000	0.280, 0.398	0.163, 1.000
Toll-like receptor 6	-0.234, 0.952	0.476, <0.001	0.521, <0.001	0.574, <0.001

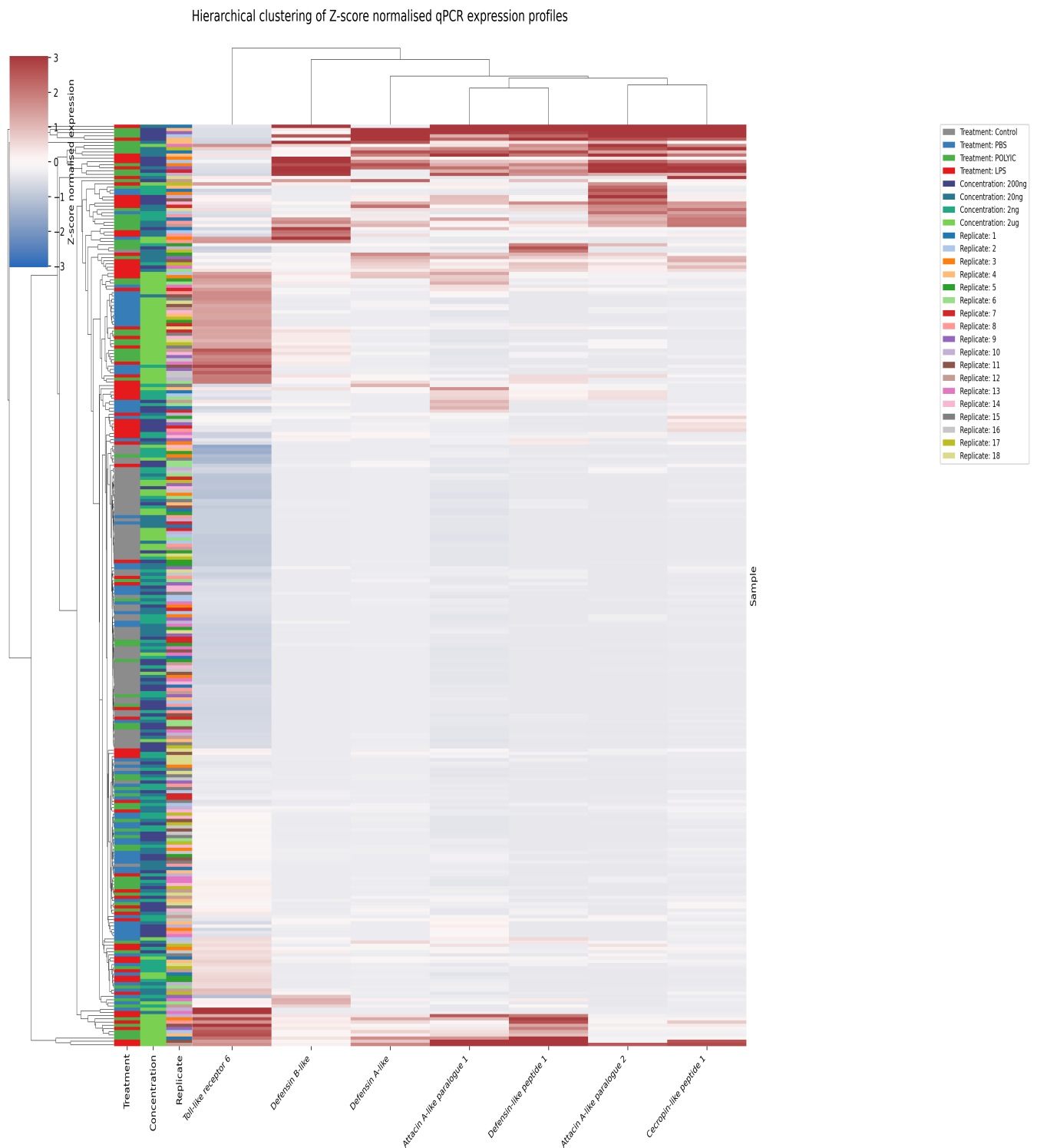


Figure 4.54 Hierarchical clustering of Z-score normalised qPCR gene expression profiles. Gene expression values were Z-score normalised across all samples prior to analysis. Hierarchical clustering was performed using Ward's linkage method to group samples with similar transcriptional profiles. Annotation bars indicate treatment, stimulant concentration and biological replicate. Red indicates relatively higher expression, and blue indicates relatively lower expression following Z-score normalisation.

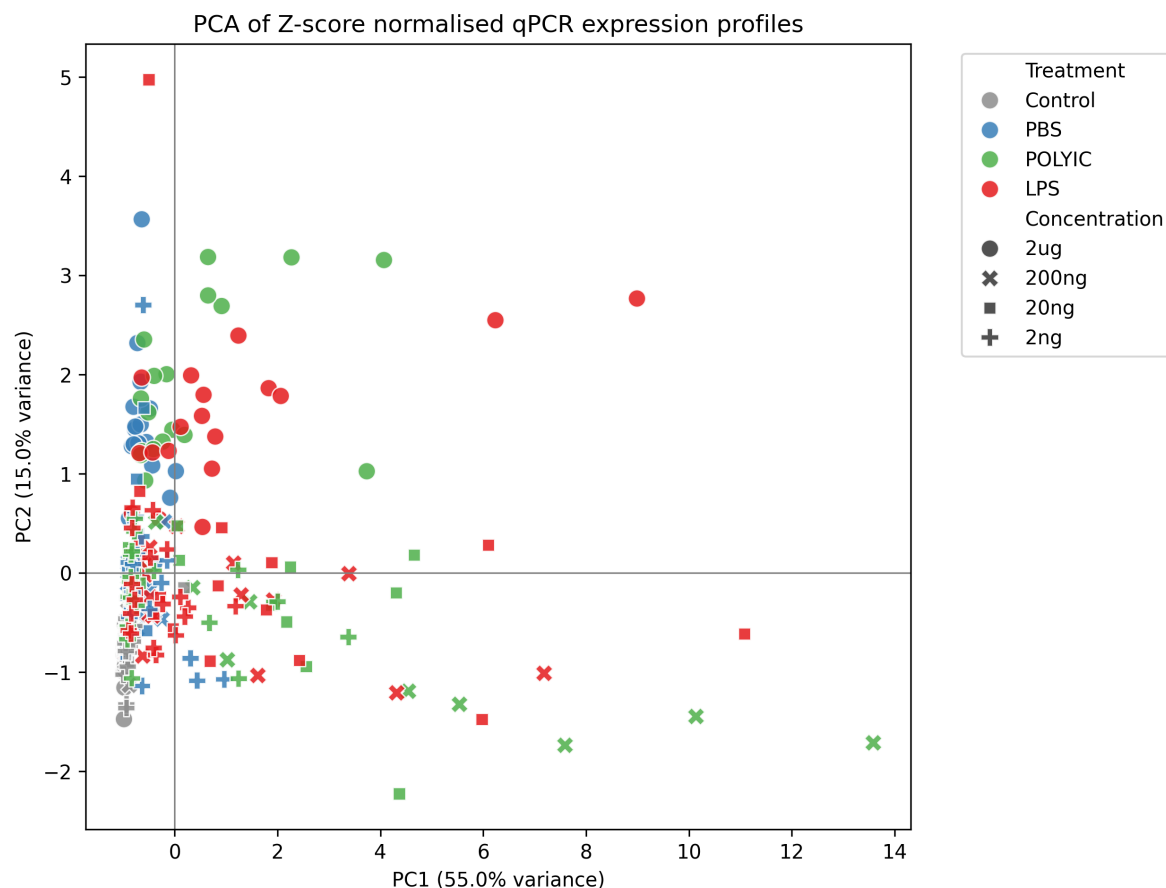


Figure 4.55 Principal component analysis (PCA) of Z-score normalised qPCR gene expression profiles. Samples are coloured according to treatment, and symbol shape indicates stimulant concentration. Principal component 1 (PC1) explained 55.0% of the total variance, while principal component 2 (PC2) explained 15.0%, accounting for 70.0% of the overall variation in gene expression.



Figure 4.56 Mean Z-score normalised gene expression profiles for the four hierarchical expression clusters. Heatmap values represent the mean Z-score normalised expression of each antimicrobial peptide gene and Toll-like receptor 6 within each cluster. Red indicates relatively higher expression and blue indicates relatively lower expression compared with the overall dataset.

Table 4.20 Summary of statistical analyses assessing the influence of treatment, stimulant concentration, biological replicate and dose on hierarchical clustering and principal component analysis (PCA) of qPCR gene expression profiles. Kruskal–Wallis tests were used for categorical metadata, Spearman's rank correlation was used for dose, and chi-square tests were used to assess associations between hierarchical cluster membership and metadata variables.

Analysis	Statistical test	Test statistic	P value	Interpretation
Treatment vs PC1	Kruskal–Wallis	H = 147.22	<0.001	Significant
Treatment vs PC2	Kruskal–Wallis	H = 78.13	<0.001	Significant
Concentration vs PC1	Kruskal–Wallis	H = 1.72	0.633	Not significant
Concentration vs PC2	Kruskal–Wallis	H = 49.55	<0.001	Significant
Replicate vs PC1	Kruskal–Wallis	H = 12.49	0.769	Not significant
Replicate vs PC2	Kruskal–Wallis	H = 21.64	0.199	Not significant
Dose vs PC1	Spearman correlation	$\rho = 0.069$	0.244	Not significant
Dose vs PC2	Spearman correlation	$\rho = 0.303$	<0.001	Significant positive correlation
Cluster membership vs Treatment	Chi-square	$\chi^2 = 62.56$ (df = 9)	<0.001	Significant association
Cluster membership vs Concentration	Chi-square	$\chi^2 = 74.83$ (df = 9)	<0.001	Significant association
Cluster membership vs Replicate	Chi-square	$\chi^2 = 47.24$ (df = 51)	0.624	Not significant

4.45. Discussion

In this chapter we quantified mRNA abundance of antimicrobial peptides (AMPs) and pathogen recognition receptors (PRRs) in immune challenged *Hermetia illucens* (The Black Soldier Fly; BSF) larvae in response to two pathogen-associated molecular patterns (PAMPs): lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid [poly(I:C)]. In parallel, we performed in silico analyses to predict signal-peptide cleavage sites, delineate the resulting mature polypeptides, to provide a predicted structural framework of the targets analysed.

Across three paralogues: Defensin-A-like (XM_038070404.1), Defensin-B-like (HERIL_Hill_BB_C34351), and Defensin-like peptide 1 (HERIL_Hill_BB_C7176), the in-silico predications consistently support the annotation of secreted, cysteine-rich insect defensins processed from larger precursors into compact mature peptides. Specifically, (i) SignalP-5.0 identifies strong N-terminal Sec/SPI signal peptides with cleavage at residues ~19-20 (ii) ProP detects a single, above-threshold proprotein convertase site near R58 with dibasic/furin R-K-[K/R]-R↓ motif (RAKR/RTKR); (iii) AMP Scanner V2 classified each as antimicrobial with near-maximal probability; and (iv) AlphaFold/ColabFold models of the predicted mature protein, yielding a compact structure comprising a short α -helix packed against an antiparallel β -sheet with cysteine spacing, consistent with the cysteine-stabilised $\alpha\beta$ (CS $\alpha\beta$) defensin fold.

Defensins are a conserved family of small, cationic host-defence peptides found across a wide variety of species, from vertebrates, invertebrates, amoeba and plants (Xu & Lu, 2020) (Lehrer & Ganz, 2002) (Thomma, Cammue, & Thevissen, 2002). In 1983, the first mammalian defensin was discovered in rabbit macrophages by Selsted and colleagues (Selsted, Brown, DeLange, & Lehrer, 1983), which shortly followed by the extractions of three human defensins in 1985 (Ganz, Daher, Bainton, & Lehrer, 1985). The common consensus from these early findings where that these defensins (also known as human neutrophil peptides [HNPs]), exhibited strong antimicrobial activity against viral, bacterial (gram +/gram-), and fungal pathogens.

In arthropods, antibacterial peptides with defensin-like properties were identified soon after (Dimarcq, Zachary, Hoffmann, Hoffmann, & Reichhart, 1990). A landmark study in the flesh fly *Phormia terranova* showed strong induction of defensin (and dipterin) following immune challenge, cementing defensins as inducible effectors of insect innate immunity, with Defensin A later following structural work from the same species, which showed a compact cysteine-stabilised $\alpha\beta$ (CS $\alpha\beta$) motif: a short α -helix packed against an antiparallel β -sheet, locked by three disulfide bonds (Cornet B. , et al., 1995).

Functionally, defensins do more than punch holes in bacterial membranes. They can target cell envelopes, inhibit intracellular processes, neutralize enveloped viruses, and modulate host inflammation, a breadth now summarised across several recent reviews (Shafee, Lay, Phan, Anderson, & Hulett, 2017) (Nagib, et al., 2025).

These small, positively charged peptides embed into hydrophobic regions of lipid membranes, subsequently causing membrane destabilisation and cell death (Lai & Gallo, 2009). Beyond membrane destabilisation, defensins also target intracellular processes (e.g., nucleic-acid binding and inhibition of macromolecular synthesis (Li, et al., Antibacterial and immunomodulatory activities of insect defensins-DLP2 and DLP4 against multidrug-resistant *Staphylococcus aureus*, 2017)), exhibit direct antiviral actions (from capsid stabilization/uncoating blockade to early post-entry inhibition (Nguyen, Nemerow, & Smith, 2010) (Bharucha, Sun, Lu, Gartner, & Garzino-Demo, 2021)), and modulate host immunity by recruiting CCR6⁺ dendritic/T cells and activating myeloid cells via TLR1/2 (Yang, et al., 1999) (Funderburg, et al., 2007). They can neutralize endotoxin, shape mucosal microbiota (Salzman, 2010) (including HD-6 “nanonets” that entrap bacteria (Chu, et al., 2012)), accelerate wound repair through keratinocyte migration and pro-angiogenic signalling (Takahashi, et al., 2021), and display anti-biofilm activity against clinically relevant pathogens (Sutton & Pritts, 2014).

Insects, like other animals, rely on an effective innate immune system to control invading microbes. For Black Soldier Fly larvae (BSFL), which develop in microbe-dense substrates, their survival depends on rapidly deployable defence modules such as antimicrobial peptides (AMPs). Since their first description in the 1980s in two dipterans

(*Sarcophaga peregrina* and *Phormia terranova*), defensins (or defensin-like peptides) have been reported across nearly all insect groups examined, including *Diptera*, *Hymenoptera*, *Hemiptera*, *Coleoptera*, *Lepidoptera*, and *Phthiraptera* (University of Nebraska Medical Centre, 2025). Their importance in vivo is supported by genetic studies; for example, dsRNA knockdown of mosquito defensin revealed that this peptide is required for defence against Gram-positive bacteria (Blandin, et al., 2002). While gram-positive responses appear to be the most common initiator of defensins, they still exhibit responses to gram-negative, antiviral, and antifungal pathogens, which is also supported in our observations (Fahmy, et al., 2024) (Hwang, et al., 2009) (Lamberty, et al., 1999). Taken together our results support the fact that defensins are wide-ranging and fast-acting effectors that complement cellular responses within the innate system. Defensins are also evolutionarily versatile. A study suggested that the Scorpion K⁺-channel toxins (α -KTx) could have evolved from a defensin-type peptide with the same CS α β scaffold (Zhu, et al., 2014). In practical terms, it underscores why mapping defensin sequences and structures in BSFL is valuable for both fundamental immunology and potential biotechnological applications.

Defensin-A-like showed the strongest and most consistent upregulation, with significant transcriptional elevation in response to both LPS and poly(I:C) across all tested concentrations (2 μ g, 200ng, 20ng, 2ng), and clear statistical distinction from PBS-treated controls ($p < 0.0001$). The magnitude and breadth of the Defensin-A-like response may support its role as a robust frontline effector capable of responding to diverse immune challenges.

In contrast, Defensin-B-like demonstrated more selective and concentration-dependent expression. It was significantly upregulated by both LPS and Poly (I:C) ($p < 0.001$ and $p < 0.01$, respectively), with a markedly stronger transcriptional induction at 2 μ g, consistent with a dose-threshold effect.

Defensin-like peptide 1, like the other two paralogues, showed strong and significant transcriptional elevation in response to both PAMPs ($p < 0.0001$). Tighter responses

were seen in the case of LPS, with dose-dependent responses seen at the higher concentrations (2µg and 200ng).

Across all three paralogues, higher concentrations of LPS and poly(I:C) were more likely to elicit stronger expression, suggesting dose-responsiveness and a scalable immune activation mechanism. These patterns align with reports in other dipterans where multiple defensin paralogues are co-regulated but serve partially divergent functions depending on tissue type, microbial target, or infection context (Lowenberger, et al., 1995). Comparable paralogue-resolved evidence in *H. illucens* is sparse, therefore, our data contributes to a field still in its early stages, and will need future functional validation of paralogue defensins in *Hermetia illucens*.

Attacins differ fundamentally from defensins in both processing logic and biophysical action. Whereas defensins are cysteine-stabilised αβ (CSαβ) mini-proteins released after a conserved furin-type cut, attacins are glycine-rich proteins whose antimicrobial region resides in an elongated, β-sheet-rich domain (Zhou, Meng, Zhu, Ma, & Chen, 2024). These typically consist of signal-peptide-bearing pre-proteins that are processed to a mature antimicrobial region; they remain best known for targeting gram-negative/positive bacteria and the OMP biogenesis pathway (Mahlpuu, Håkansson, Ringstad, & Björn, 2016). Therefore, attacins primarily cause pathway-level disruption that alters permeability and inhibits growth rather than causing lysis.

Attacins were first reported in immune-challenged *Hyalophora cecropia* (cecropia moth) as part of the classic surge of inducible haemolymph antimicrobials discovered in the early 1980s (Hultmark, et al., 1983). Early characterisation established attacins as glycine-rich proteins active primarily against Gram-negative bacteria. They uncovered a distinctive mechanism: rather than classic membrane lysis, purified attacin inhibits the synthesis of outer-membrane proteins (OMPs) in *Escherichia coli* by interfering with omp gene transcription and lowering omp mRNA levels (Carlsson, Engström, Palva, & Bennich, 1991). This produces secondary membrane defects and growth inhibition without broad cytolysis, distinguishing attacins mechanistically from many cationic AMPs.

Two Attacin-A-like paralogues from *Hermetia illucens* were analysed to evaluate whether structural divergence corresponded to differential immune responsiveness following PAMP challenge. In silico screening using SignalP-5.0 predicted strong N-terminal Sec/SPI-type signal peptides (cleavage ~AA20), and AMP Scanner v2 classified both paralogues as antimicrobial with maximal confidence. However, only Paralogue 2 contained a high-confidence R-X-[K/R]-R↓ convertase motif (RARR↓), while Paralogue 1 lacked such a site entirely, necessitating a conservative mature boundary definition from the signal-peptidase cut. Structural modelling supported this divergence: both proteins adopted β-sheet-rich folds typical of attacins, but Paralogue 2 displayed a more compact domain downstream of its RARR motif.

We observed expression profiles differing strongly across all treatments and concentrations (Kruskal–Wallis, $p < 2.2 \times 10^{-16}$). RT-qPCR analysis of both attacin paralogues saw a relatively different transcriptional profile to one another. Paralogue 1 (Hill_BB_C16882|attacin) had a dose-dependent response ($2\mu\text{g} > 200\text{ng} > 20\text{ng} > 2\text{ng}$) in LPS with high statistical confidence ($p = < 0.0001$ to the PBS control). Poly(I:C) on the other hand saw no statistical relevance captured against control groups. This profile is IMD-consistent (Gram-negative mimic). The more specific response in paralogue 1 (Hill_BB_C10074), a transcript already validated as an antimicrobial peptide against *Pseudomonas aeruginosa* (Fahmy, et al., 2023), positions it as a key player for microbial responses and improving larval resilience.

Paralogue 2 (Hill_BB_C10074|attacin-like) on the other hand had a much varied and broad response across all concentrations ($p = < 0.0001$). Median expression tended to be slightly higher under LPS, especially at lower doses, indicating greater sensitivity to Gram-negative cues. The combination of higher medians, wider range, and consistent upregulation suggests tighter immune regulation and/or more efficient transcript accumulation for this paralogue. The LPS-favoured responses seen again in Paralogue 2, align with canonical IMD-pathway control of glycine-rich AMPs and with the historical mechanism of attacins acting against Gram-negative bacteria by inhibiting OMP

synthesis. Unlike paralogue 1, we see here broad ranging Poly(I:C) inducibility, consistent with pathway cross-activation.

This data showed dose-scalable induction, indicating that BSFL attacins mount rapid, flexible defences against the Gram-negative burden typical of organic-waste substrates, but like in the case of paralogue 2, these responses can be extended to viral mimics like Poly (I:C), indicating viral responses can also be maintained through attacin expression, perhaps through crosstalk through multiple immune modules. Together, this data suggests functional divergence of attacin paralogues and an AMP programme not confined to Gram-negative cues.

The last for the antimicrobial peptides (AMPs) analysed was cecropin-like 1 (Hill_SB_C698|cecropin), one of the first insect AMPs discovered, and isolated from the giant silk moth *Hyalophora cecropia* (Steiner, Hultmark, Engström, Bennich, & Boman, 1981). These small, secreted, cationic peptides kill microbes by targeting membranes and were shown to be widely expressed in *Diptera* and *Lepidoptera*, where they form a major arm of humoral immunity (Wu, Patocka, & Kuca, 2018). In *Hermetia illucens*, (The Black Soldier Fly; BSF), we found that cecropin-like 1 was present and inducible via pathogen associated molecular patterns LPS and Poly (I:C).

Our in-silico analyses strongly support the classification of this sequence as a secreted AMP, consistent with the typical features of cecropin-like peptides (signal peptide, amphipathic α -helix, and high AMP prediction probability). Previous structural analysis of cecropin peptides have discovered their structure to form two α -helices separated by a flexible Gly/Pro-rich hinge in the membrane-mimetic environments, whereas some members present as a single continuous helix; many are disordered in water and fold upon membrane contact (citation?). Our ColabFold model of the BSF cecropin-like peptide converged to one amphipathic α -helix in aqueous prediction, which is compatible with the family's known behaviour and does not exclude a helix-hinge arrangement upon membrane binding (Lee, et al., 2013) (McDonald, Jones, Plate, Meiler, & Gulsevin, 2023).

Cecropins are typically ~29-42 amino acids (aa), disulfide-free and form amphipathic α -helices that are unstructured in water but fold upon contacting lipid bilayers; the N terminus is strongly cationic/amphipathic and the C-terminus more hydrophobic, an arrangement that promotes membrane binding and disruption (Sato & Feix, 2006).

Across insects, cecropins have shown broad antibacterial activity and additional antiviral/anti-biofilm effects described for some family members (Feng, et al., 2020) (Peng, et al., 2024). Cloning and functional characterisation were performed on CecropinZ1 in BSF providing one of the first experimental validations for the species (Elhag, et al., 2017). In vivo and transcriptomic studies show that cecropin genes are rapidly induced in fat body and haemocytes by infection and are largely controlled by the Imd/NF- κ B pathway (with Toll crosstalk), reinforcing their role as frontline humoral effectors (Tingvall, Roos, & Engström, 2001). Beyond classical antibacterial activity, multiple reports document antiviral effects (e.g., cecropin A inhibiting several human viruses in cell systems) and anti-biofilm properties; engineered or D-enantiomer variants can display improved stability and biofilm disruption *against Pseudomonas aeruginosa* (Wang, et al., 2022). Comprehensive reviews summarize these activities and the therapeutic potential of natural and synthetic cecropins, while noting typical peptide-drug challenges (stability, salt/serum effects) (Brady, Grapputo, Romoli, & Sandrelli, 2019). Hill-Cec1, Hill-Cec10 were also determined to exhibit anti-gram-negative activity, rapid kill kinetics, outer/inner-membrane permeabilization, and anti-biofilm effects with low haemolysis, reinforcing that BSF cecropins behave as classical amphipathic, membrane active peptides (Van Moll, et al., 2022).

Hermetia illucens possesses an unusually expansive cecropin repertoire, recently resolved in a genome-anchored survey. They identified 33 cecropin genes, with 32 clustered on a single chromosome, which were typically organised as two-exon loci. Often, when very similar genes sit next to each other on the same chromosome, it usually means they arose by local duplication, revealing tandem duplicates that encode identical mature peptides, yielding 15 distinct mature cecropins at the peptide level (Kalsy, et al., 2020). Synthetic testing of these peptides showed broad anti-gram-negative activity with low haemolytic risk, and highlighted Cecropin H3 as a particularly

potent candidate (Minimum Inhibitory Concentration [MIC] 4 µg/mL against *Escherichia coli*) with rapid kill kinetics and anti-biofilm effects (Peng, et al., 2024).

Our RT-qPCR showed significant induction of the cecropin-like 1 gene at 24 h, with poly(I:C) and LPS responses exceeding the PBS injection baseline. This mirrors canonical insect immunity in which Imd/Toll axes drive cecropin transcription after Gram-negative and viral-mimic stimuli and is consistent with BSF reports of highly inducible AMP repertoires. LPS appears to elicit a more specific response than Poly(I:C). Within each LPS dose the IQRs are narrow and the whiskers short, so most larvae cluster tightly around a higher median expression ($p = 0.0001$). By contrast, Poly (I:C) shows wider dispersion with higher variability between larva.

Across insects, antimicrobial peptide (AMP) transcripts rise within hours of challenge and often remain elevated into the 24-48h window, depending on pathway, tissue, and AMP family (Lemaitre, Reichhart, & Hoffmann, *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms, 1997). Time-course studies in *Drosophila* show rapid induction via Imd/Toll-NF-κB with strong signals by 6-12h, with lower, but long-lasting effects existing at 24 h, 48h, and some even 72h. Thus, the elevated AMP levels we observe at 24 h are compatible with a long-lasting immune programme that persists beyond the immediate early response. Together, these data support that BSF antimicrobial responses offer sustained AMP activation to complement their robust innate immune responses. In this study, we also screened Hill_BB_C1290|dipteracin, Hill_BB_C9109|dipteracin, Hill_SB_C2730|cecropin, and toll-like receptor 7 [*Hermetia illucens*] (LOC119654889; XM_038060505.1), but we found no evidence of mRNA signal at the 24-hour mark, which may reflect earlier induction/decline, rather than absence of responsiveness.

While individual gene analyses demonstrated sustained antimicrobial peptide induction following immune stimulation, the complementary multivariate analyses performed in this chapter revealed that these responses were highly coordinated at the transcriptional level. Heatmap visualisation, hierarchical clustering and principal component analysis consistently grouped samples according to their immune

expression profiles rather than technical replicate or batch, demonstrating that immune stimulation was the dominant source of transcriptional variation. Four major transcriptional response patterns were identified, comprising a Defensin B-like-enriched response, a broad antimicrobial peptide activation profile, a low-expression baseline state, and a TLR6-associated response characterised predominantly by higher-dose stimulation. Together, these findings demonstrate that BSFL immunity operates through coordinated transcriptional programmes rather than independent regulation of individual immune genes.

(TLR6), was the only receptor we could detect at 24 hours, (albeit low-level signals). Our in-silico analysis using InterPro/Phobius mapping, exhibits the textbook Toll/TLR layout: a long LRR-rich (leucine-rich) ectodomain (res. ~1–949), a single-pass transmembrane helix (~950–973), and a cytosolic tail with a Toll/Interleukin-1 Receptor (TIR) domain (~1,000–1,120). LRR solenoids form a curved “horseshoe” scaffold that provides broad protein-ligand binding surfaces in many recognition receptors; in TLRs, these surfaces accommodate microbial patterns or endogenous ligands/co-ligands (Bella, Hindle, McEwan, & Lovell, 2008). The TIR domain is the intracellular signalling module that recruits adaptors (e.g., MyD88-family proteins) to nucleate kinase complexes and drive NF- κ B/Rel-type transcriptional responses (in insects, MyD88-Tube-Pelle downstream of Toll; in mammals, MyD88/TRIF) (Horng & Medzhitov, 2001).

TLR6 is a type 1 membrane Toll-like receptor, in mammals it functions primarily as a TLR2/6 heterodimer, and classically recognizes diacylated bacterial lipopeptides (e.g., MALP-2, Pam2CSK4) with co-receptors and accessory proteins and signalling via MyD88>IRAKs>NF- κ B/MAPK to induce inflammatory genes. This division of labour: TLR1/2 for triacyl and TLR2/6 for diacyl lipopeptides, is a core feature of mammalian TLR biology (El-Zayat, Sibaii, & Mannaa, 2019).

In insects, literature shows how Toll/TLR signalling is conserved and inducible, showing upregulation after viral and bacterial challenge (Liu, Wei, & Wang, 2021) (He, et al., 2023). A full repertoire of Toll-like receptor, including TLR6 were seen to be upregulated in the gypsy moth *Lymantria dispar*, upon immune stimulation of the baculovirus,

Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV), showing expression of Toll-like receptor 6 (TLR6) up to 6 days.

At 24 h, RT-qPCR of TLR6 transcripts were significantly higher than both no-treatment and PBS controls, with the strongest induction under LPS; however, the absolute copy numbers remain low, indicating that the effect size at this timepoint is modest despite statistical significance. Although classical LPS sensing is TLR4-dependent in mammals, in insects LPS/Gram-negative cues drive robust innate cascades (often via IMD) that can secondarily up-regulate Toll/TLR components; Toll-pathway cross-activation is well documented (Valanne, Wang, & Rämet, 2011). The comparatively tighter dispersion in the LPS boxes suggests a more specific, uniform transcriptional program across larvae, whereas Poly I:C shows wider spread and only a modest elevation at some doses, consistent with heterogeneous engagement of antiviral sensors and downstream crosstalk, rather than direct TLR6 ligation.

The additional regression, fold-change and dose-response analyses further refined these observations by demonstrating that treatment was the principal determinant of transcriptional activation across all immune targets. Absolute copy number and fold-change analyses confirmed that antimicrobial peptides underwent substantially greater transcriptional induction than TLR6, while linear modelling identified treatment as a significant predictor of expression for every target gene. In contrast, statistically significant dose-dependent relationships were comparatively restricted, with Defensin B-like and TLR6 showing the strongest evidence of concentration-dependent regulation. These findings indicate that the presence of an immune stimulus exerts a greater influence on transcriptional activation than increasing PAMP concentration alone.

In conclusion, these findings demonstrate that the innate immune response of *Hermetia illucens* is characterised by sustained, coordinated and functionally specialised transcriptional activation following immune stimulation. While individual antimicrobial peptide families displayed distinct expression profiles, complementary multivariate analyses demonstrated that these genes operate as integrated transcriptional programmes that are primarily driven by immune treatment rather than

technical or biological variation. Although statistically significant dose-dependent regulation was identified for only a subset of genes, particularly Defensin B-like and TLR6, immune stimulation consistently elicited robust activation across the broader antimicrobial peptide repertoire. Together, these findings support a model in which BSFL deploy a flexible and highly coordinated innate immune response, combining broad-spectrum antimicrobial peptide activation with more selective pattern-recognition receptor regulation. Within the context of *Hermetia illucens*, which possesses one of the largest characterised AMP repertoires among Diptera, this work provides one of the first integrated analyses of antimicrobial peptide expression in *Hermetia illucens*, combining transcript abundance, fold-change analysis, dose-response modelling and systems-level transcriptional profiling within a single experimental framework. Collectively, these findings establish a valuable framework for future functional studies investigating immune signalling, antimicrobial peptide biology and pathogen control in Black Soldier Fly larvae.

Chapter 5. Phage-Based control of *Salmonella enterica* Serovar Typhimurium SL1344 in *Hermetia illucens* using the SPFM14

5.1. *Salmonella enterica* as a challenge in insect production systems

The Black Soldier Fly (*Hermetia illucens*) has emerged as one of the most commercially significant insect species globally, with applications spanning waste bioconversion, animal feed production, fertiliser generation, and the recovery of high-value bioactive compounds (Bukchin-Peles, Lozneva, Tomberlin, & Zilberman, 2025). As industrial-scale production continues to expand, maintaining microbial safety throughout the production chain has become increasingly important. While Black Soldier Fly Larvae (BSFL) possess a robust innate immune system and are capable of reducing the abundance of several microorganisms within organic waste streams, this capacity is thought to be mediated not only by host immune defences, including antimicrobial peptide production, but also by a diverse and dynamic gut microbiome (Zhang, et al., 2025). The BSFL gut microbiota contributes to nutrient acquisition, waste degradation, colonisation resistance, and immune regulation, collectively supporting larval resilience in microbially dense environments (Ment & Mishra, 2025). Interactions between the host immune system and resident microbial communities are therefore likely to play an important role in determining whether invading pathogens can successfully establish and persist within the larval gut.

Salmonella enterica is among the leading causes of bacterial foodborne disease worldwide and represents a major challenge for both agricultural and food production systems (World Health Organization, 2018). The bacterium is capable of colonising a wide range of hosts and environmental niches, contributing to its persistence throughout food production chains. In livestock systems, *Salmonella* contamination can result in significant economic losses, reduced consumer confidence, product recalls, and substantial public health risks. Consequently, the control of *Salmonella* remains a priority across animal production industries.

Although BSFL possess several mechanisms that may contribute to pathogen suppression, including antimicrobial peptide production, gut physicochemical barriers, microbial competition, and microbiome-mediated colonisation resistance, these

protective effects do not necessarily guarantee complete pathogen elimination. Previous studies have demonstrated that BSFL can significantly reduce *Salmonella* abundance during waste conversion and manure treatment processes (Erickson, Islam, Sheppard, Liao, & Doyle, 2004) (Lalander, Fidjeland, Diener, Eriksson, & Vinneras, 2015). However, more recent investigations have reported variable outcomes, with pathogen persistence influenced by factors such as substrate composition, environmental conditions, bacterial strain, and initial contamination level (De Smet, Vandeweyer, Van Moll, Lachi, & Van Campenhout, 2021). These findings suggest that the presence of BSFL alone should not be assumed to provide complete protection against foodborne pathogens entering insect production systems.

As BSFL become increasingly incorporated into animal feed production chains, understanding the factors that govern pathogen establishment, persistence, and control within the larval host becomes increasingly important. Consequently, there is growing interest in developing targeted biocontrol strategies capable of complementing the natural antimicrobial capacity of BSFL and improving microbiological safety throughout insect production systems. Among these approaches, bacteriophage therapy has emerged as a promising candidate due to its host specificity, self-amplifying nature, and ability to target antimicrobial-resistant bacteria while minimising disruption to beneficial microbial communities (Girma, 2025). Within insect production systems, phage-based interventions may therefore provide a targeted method of reducing pathogen burden without negatively impacting beneficial members of the larval microbiota.

Building upon the transcriptomic characterisation of immune-related genes presented in Chapter 3 and the immune activation responses described in Chapter 4, this chapter extends the investigation from immune stimulation to a biologically relevant host-pathogen system involving *Salmonella* infection and bacteriophage intervention. Despite growing interest in microbial safety within insect production systems, controlled in vivo models investigating *Salmonella* persistence and biological control within BSFL remain comparatively limited.

The present chapter therefore sought to establish and characterise an in vivo *Salmonella enterica* serovar Typhimurium (SL1344) infection model in *Hermetia illucens* larvae and evaluate the potential of bacteriophage SPFM14 as a biological control agent. SPFM14 is a lytic *Salmonella* bacteriophage previously identified as possessing strong activity against *Salmonella enterica*, making it suitable for evaluating phage-mediated pathogen reduction within BSFL. Specifically, this work investigated the ability of SL1344 to colonise and persist within BSFL, assessed the impact of prophylactic and concurrent phage treatment on bacterial burden, and examined phage persistence within the larval host. Together, these experiments provide a foundation for understanding pathogen-phage-host interactions in BSFL and inform the development of future disease management strategies for industrial insect production systems.

5.2. Bacteriophages: From Discovery to Biological Control

Bacteriophages, or simply phages, are viruses that specifically infect and replicate within bacterial hosts. Discovered independently by Frederick Twort in 1915 and Félix d'Hérelle in 1917, bacteriophages are considered the most abundant biological entities on Earth, with an estimated 10^{31} particles in diverse environments, including soil, water, and within living organisms (Twort, 1915), (d'Herelle, 1917), (Batinovic, et al., 2019). They exhibit remarkable host specificity, often infecting only a single bacterial species or even a specific strain (Clokier, Millard, Letarov, & Heaphy, 2011). This precision, coupled with intrinsically bactericidal lytic cycles, makes bacteriophage therapy an attractive targeted antimicrobial that can remove pathogens while minimising disruption to commensal communities (Subramanian, 2024) (Febvre, et al., 2019).

Historically, phage therapy gained momentum for its therapeutic use in clinical medicine before the antibiotic era. Following the advent of penicillin in 1928, bacteriophage therapy largely receded in western medicine in the 1940s, while continuous practice persisted in parts of the former Soviet Union and Eastern Europe, most notably at the Eliava Institute in Tbilisi, Georgia, and the Hirsfeld Institute in Wrocław, Poland (and, to a lesser extent, in Russia and Lithuania) (Wittebole, De Roock,

& Opal, 2014). Interest has since resurged worldwide as antibiotic resistance has grown, offering a promising solution not only in clinical medicine but also in agriculture, aquaculture, food safety, and environmental microbiology (Ahmed, et al., 2024).

Antimicrobial resistance (AMR) erodes the effectiveness of antibiotics across human, veterinary and food-production systems, and is recognised as a global health issue. In forecasts to 2050, GBD (Global Burden of Disease Study) 2021 estimates 1.91M attributable and 8.22M associated AMR deaths per year globally, with the heaviest rates in South Asia and Latin America/Caribbean, a shift toward older adults, and large mortality under improved care and Gram-negative drug scenarios (Antimicrobial Resistance Collaborators, 2021).

To tackle the rise in AMR, phage-based approaches are being explored in clinical therapy (including personalised/compassionate use), food safety (as processing aids to reduce surface contamination), aquaculture and agriculture, and as components of biocontrol strategies in complex microbiomes (Ahmed, et al., 2024).

5.3. Biology of Infection: Lytic Killing and Lysogenic Persistence

Derived from the Greek phagein (“to devour”), bacteriophages attach to specific receptors on the bacterial surface and inject their genome into the cell. In contractile-tailed (myovirus-like) phages, the baseplate engages the cell, the tail sheath contracts like a syringe, and the tail tube pierces the envelope to deliver genetic material (other tailed phages achieve entry without sheath contraction, but all rely on precise receptor recognition).

Once inside, lytic phages rapidly hijack host metabolism. Early phage genes redirect transcription, often by modifying or functionally replacing host RNA polymerase, so that viral mRNA is preferentially produced. Middle and late genes then drive genome replication and assembly of the capsid and tail. Finally, a holin-endolysin system

permeabilises the membrane and degrades the cell wall, causing lysis and release of progeny virions that can infect neighbouring cells.

By contrast, temperate phages can enter lysogeny: the injected genome integrates into the bacterial chromosome as a prophage and most phage genes are silenced. The host continues to divide, vertically transmitting the prophage to its descendants. Induction, for example, the SOS response to DNA damage, nutrient starvation, or exposure to certain antibiotics, reactivates the prophage, switching to the lytic programme and producing new virions.

Two features of this biology are central to phage use as antibacterials: (i) strict host specificity, determined by receptor binding and early control of host transcription; and (ii) the bactericidal outcome of the lytic cycle. Together, they underpin current interest in bacteriophages as precision agents in the era of antimicrobial resistance (AMR) (Sharma, Singh, Sharma, Verma, & Ratn, 2025). As SPFM14 is a lytic bacteriophage, the bactericidal processes associated with the lytic cycle are of particular relevance to the present study.

5.4. The AMR Imperative for Phage-Based Interventions

Antimicrobial resistance (AMR) is a growing global health crisis that threatens the effective treatment and prevention of bacterial infections (Marston, Dixon, Knisely, Palmore, & Fauci, 2016). It occurs when microorganisms such as bacteria, viruses, fungi, and parasites evolve mechanisms to withstand the drugs designed to kill them. Of particular concern is the rise in antibiotic-resistant bacteria, which has been accelerated by the widespread and often indiscriminate use of antibiotics in human medicine, agriculture, and animal production systems (Kardas, Devine, Golembesky, & Roberts, 2005).

The World Health Organisation has identified AMR as one of the top ten global public health threats facing humanity (Figueras, 2024). Resistant infections not only lead to

prolonged illness and increased mortality but also impose significant economic burdens on healthcare systems worldwide (Poudel, et al., 2023). Without urgent action, it is estimated that AMR could cause 10 million deaths annually by 2050, costing the global economy trillions of dollars (O'Neill, 2014).

Using >23 million infections from 104 countries, the 2025 WHO GLASS report estimates that ~1 in 6 laboratory-confirmed bacterial infections worldwide is antibiotic-resistant, with markedly higher levels in South-East Asia and the Eastern Mediterranean and lower levels in Europe and the Western Pacific. Resistance in Gram-negative pathogens is rising fastest (notably carbapenem resistance in *K. pneumoniae*, *E. coli*, *Acinetobacter*, and fluoroquinolone resistance in non-typhoidal *Salmonella*), while MRSA remains prevalent. Countries with limited surveillance and lower UHC (Universal Health Coverage) show higher reported resistance, reflecting both true burden and sampling bias (World Health Organization, 2025).

5.5. Regulatory and Industrial Deployment of Bacteriophages

The successful application of bacteriophages across clinical, veterinary, food and agricultural systems has demonstrated their potential as targeted biological control agents. Numerous studies have reported effective reduction of bacterial pathogens including *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus spp.*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Salmonella spp.*, *Acinetobacter baumannii*, *Vibrio cholerae*, *Shigella spp.*, and *Aeromonas hydrophilia*, while maintaining favourable safety profiles (Smith & Huggins, 1982), (Smith, Huggins, & Shaw, 1987), (Capparelli, Parlato, Borriello, Salvatore, & Iannelli, 2007), (Biswas, et al., 2002), (Vinodkumar, Neelagund, & Kalsurmath, 2005), (Hagens, Habel, Von Ahsen, Von Gabain, & Bläsi, 2004), (Loc Carrillo, et al., 2005), (Khan & Rahman, 2022) (Thanki, et al., 2022), (Elshamy, et al., 2025), (Yen, Cairns, & Camilli, 2017), (Mai, Ukhanova, Reinhard, Li, & Sulakvelidze, 2015), (Muliya Sankappa, et al., 2024). Consequently, the primary challenge facing phage therapy is no longer proof-of-concept efficacy, but the

development of robust regulatory frameworks capable of supporting routine deployment.

Regulatory acceptance of bacteriophages continues to expand internationally. In the United Kingdom patient access to bacteriophage therapy is still primarily through unlicensed, case-by-case supply, “Specials” under the Human Medicines Regulations 2012, when licensed options are unsuitable or have failed, with clinical liability and governance remaining with the NHS prescriber and Trust (GOV.UK, 2025). Across Europe, several countries have implemented pragmatic approaches to personalised phage therapy, including Belgium’s magistral preparation framework and specialised clinical centres in France, Germany, and Poland (Pirnay & Verbeken, 2023) (Ferry, et al., 2024). In the United States, multiple phage products have received FDA Generally Recognized As Safe (GRAS) status for food-safety applications, while compassionate-use programmes continue to support clinical implementation (Vikram, Callahan, Woolston, Sharma, & Sulakvelidze, 2022) (Soffer, Woolston, Li, Das, & Sulakvelidze, 2017). Collectively, these developments demonstrate increasing regulatory recognition of bacteriophages as viable biological interventions.

Beyond clinical medicine, bacteriophages are increasingly being applied across food-production and agricultural systems. Applications include control of foodborne pathogens during food processing, reduction of bacterial diseases in aquaculture, suppression of plant pathogens in crop production, and incorporation into antimicrobial food-packaging technologies. These approaches are particularly attractive because they provide targeted bacterial control while reducing reliance on conventional antibiotics and minimising disruption to beneficial microbial communities (Zeng, et al., 2025) (Albarella, Dall’Ara, Rossi, & Turin, 2025).

Despite these advances, several barriers continue to limit widespread adoption, including the need for harmonised regulatory pathways, standardised susceptibility testing, scalable manufacturing processes, and comprehensive pharmacovigilance frameworks. Nevertheless, the growing regulatory acceptance and successful deployment of bacteriophages across diverse sectors highlight their potential as practical biocontrol agents. While applications within food, agriculture, and clinical

medicine are increasingly well established, the use of bacteriophages within insect production systems remains comparatively underexplored, particularly in relation to pathogen management in *Hermetia illucens*.

5.6. Phages in Insects: Model Insights and Application to BSFL

Black Soldier Fly Larvae (BSFL) are generally regarded as resilient to many microbial challenges; however, a small number of bacterial species have been conclusively shown to cause disease under experimental or production-relevant conditions. Notably, *Paenibacillus thiaminolyticus* has been identified as the causative agent of a soft-rot syndrome in mass-rearing settings. This leads to intestinal damage, reduced microbial diversity, and temperature-dependent larval mortality when vegetative cells are introduced through feed (She, et al., 2023). Similarly, *Pseudomonas protegens* (Pf-5) has been demonstrated to induce dose-dependent mortality following wounding or injection (Shah, Ruan, van Loon, & Dicke, 2023). While large-scale bacterial epizootics remain uncommon, these findings highlight that BSFL are not entirely impervious to infectious disease. The capacity of specific bacteria to exploit stress or injury underscores the need for proactive health management in rearing systems and provides a biologically relevant context for exploring bacteriophage-based control strategies in insects.

Evidence from *Galleria mellonella* demonstrates that phages delivered orally or by injection can significantly reduce pathogen burden and improve host survival across multiple pathogens, including *Salmonella*, *Klebsiella pneumoniae*, *Burkholderia cepacia* complex, and *Pseudomonas aeruginosa*, while showing no inherent toxicity of well-designed cocktails (Nale, et al., 2021), (Kosznik-Kwaśnicka, et al., 2022), (Kelly & Jameson, 2024), (Quispe-Villegas, et al., 2025), (Seed & Dennis, 2009), (Jeon & Yong, 2019). Comparable tools now exist for *Drosophila melanogaster*, where standardised systemic-infection protocols allow rapid evaluation of phage efficacy and simple pharmacokinetic readouts in vivo (Jang, Bae, & Cho, 2019), (Lindberg, McKean, & Wang,

2014). As it stands, we are not aware of any bacteriophage trials tested in *Hermetia illucens*.

Translating phage research to farmed insects, *Hermetia illucens* (BSFL) systems can use substrate-mediated or feed-based phage delivery to suppress target pathogens at critical points in the cycle, prophylactically to help maintain a stable microbial community or remedially when contamination is detected. Building on this rationale, we translate these principles into *Hermetia illucens* trials, to test the efficacy of prophylactic bacteriophage treatment (applied ahead of bacterial challenge), and concurrent (phage + bacteria together), testing pathogen burden, larval health and bacteriophage shedding dynamics in the larval gut.

As it stands, we are not aware of any published studies investigating bacteriophage-mediated control of *Salmonella* within *Hermetia illucens*. Consequently, this chapter sought to establish an in vivo *Salmonella Typhimurium* SL1344 infection model in BSFL and evaluate the efficacy of prophylactic and concurrent administration of the lytic bacteriophage SPFM14. In addition, phage persistence within the larval host was assessed to determine whether retention of SPFM14 may influence the efficacy of bacteriophage-mediated pathogen control.

5.7. Methods and Materials

5.8. Black Soldier Fly Larvae (BSFL) Rearing, Handling and Ethics

Late-instar (250-300mg) Black Soldier Fly Larvae (BSFL) (*Hermetia illucens*) were obtained from Better Origin, maintained at 27°C, 70% humidity. Larvae were starved for 12 hours before injection to minimise gut content interference and were surface-sterilised using 70% ethanol. Ethical approval was carried out with AWERB 2023 12442 11402.

5.9. Bacterial and Bacteriophage strains

The phage-propagating host, *Salmonella enterica* serovar Typhimurium SL1344 (GenBank accession: FQ312003), was used in this study, a well-characterised food-borne pathogen implicated in human gastroenteritis and common in waste/organic materials. This strain was stored in 50% glycerol broth (Abtek Biologicals Ltd) at -80°C for long term use, with fresh colonies grown weekly on Xylose Lysine Deoxycholate (XLD) agar (Oxoid) for 18h at 37°C. Prior to challenge assays, one SL1344 colony was picked and grown overnight (~16h) in Luria-Bertani (LB) broth at 37°C.

To test the efficacy of *Salmonella* clearance in *Hermetia illucens*, we used the “jumbo phage” SPFM14, isolated from United Kingdom pigs (accession number: LR535908; LR535911, respectively) which were obtained from the Becky Mayer Centre for Phage Research. (Thanki, Brown, Millard, & Clokie, 2019).

5.10. Transmission Electron Microscopy (TEM) of Phage Preparations

Phage lysates were concentrated and exchanged into a volatile buffer prior to grid prep. Briefly, 1.0ml of phage suspension was transferred to a 1.5ml microcentrifuge tube and centrifuged at 21,000 x g for 60 min at 4°C. Approximately 920 µL of supernatant was removed carefully, and the pellet was resuspended in 500 µL of 0.1M ammonium acetate. These preparations were then used immediately for negative-stain TEM. Carbon-film copper grids were glow-discharged immediately before use. A 5 µL drop of concentrated phage suspension was applied to each grid for ~2 min. Excess liquid was blotted with filter paper, and the grid was briefly rinsed with ultrapure water. Grids were negatively stained with 1% uranyl acetate (two sequential 5 µL drops with gentle blotting between applications) and air-dried thoroughly before imaging. Grids were examined on a transmission electron microscope operating at 120 kV. Images were captured at ~50,000x magnification. Negative-stain TEM images were analysed in Fiji (ImageJ, v2.16.0/1.54p). Original, un-resized TIFFs exported from the TEM software were opened with Bio-Formats. Images were calibrated using the embedded 100 nm scale bar. For

each phage, 10 intact particles were measured for mean measurements of the capsid and tail length.

5.11. Pilot Study: Optimising *Salmonella enterica* Serovar Typhimurium infection, and Prophylactic bacteriophage therapy in *Hermetia illucens*

To determine successful colonisation of SL1344 in the larval gut and test the impact of prophylactic versus concurrent phage delivery on bacterial burden in *H. illucens*, we ran an initial optimisation pilot. Overnight SL1344 cultures in LB broth were centrifuged (20 min, 5,000 x g, RT), the supernatant discarded, and the pellets washed three times with 10ml of PBS (phosphate-buffered saline). The final SL1344 suspension and SPFM14 were serially diluted (1:10) to achieve matched dose tiers of 10^3 , 10^5 , or 10^7 CFU/PFU per larva delivered in 10 μ L. Larvae were surface-rinsed in 70% ethanol and injected (1ml syringe) at the third-last metamere using a Hamilton syringe. Controls were untreated and PBS-injected; treated groups received SPFM14 phages either 1 h before challenge (1-h, prophylaxis) or at the time of challenge of SL1344 (0 h, concurrent) (n=10). Larvae were incubated at 28°C for 24 hours before enumeration. Subsequently, larvae were anaesthetized on ice prior to dissection; the gut was extracted and vortexed in 500 μ L of PBS. 10 μ L of gut lysate were spotted on XLD in triplicate and grown overnight at 37°C. Black colonies were counted and calculated to determine CFU/larva of SL1344 burden. In addition, phage plaques were observed using the double agar overlay method, repeating the same 10 μ L of gut lysate in triplicate.

5.12. Identification of XLD-grown gut colonies from *Hermetia illucens* after SL1344 challenge $\pm$ SPFM14 by MALDI-TOF MS

Gut homogenates from *H. illucens* (post-challenge of $\pm$ SPFM14 and SL1344) were plated on XLD for ~16 hours at 37°C, and due to the diverse microbiome of *Hermetia*, various distinct colonies grew on the plates. Individual colonies were picked into separate 1.5 mL microcentrifuge tubes containing 300 μ L of Milli-Q water and 900 μ L of 75% ethanol to inactivate cells. Tubes were then centrifuged at 10,000 rpm for 2 min;

supernatants were discarded and pellets centrifuged again under the same conditions. Residual ethanol was removed by pipette, and pellets were air-dried for 3-5 min at room temperature until solvent had fully evaporated. Pellets were extracted by adding 10 μ L 70% (v/v) formic acid, mixing to dissolve, followed by 10 μ L acetonitrile with further mixing. Extracts were centrifuged again (10,000 rpm, 2min) and the supernatants transferred to fresh tubes. For each extract, 1 μ L was spotted onto a stainless-steel MALDI chip and air dried. Spots were overlaid with 1 mL CHCA (α -cyano-4-hydroxycinnamic acid) matrix prepared according to the manufacturer's instructions and allowed to crystallise completely. Each target included a calibration/quality-control standard (*E. coli* MG1655) and a matrix-only blank. Spectra were acquired by MALDI-TOF MS in linear positive mode over ~2-20 kDa using the instrument's default bacterial ID settings. Peak lists were matched to the reference library, and identifications were assigned using standard score thresholds

5.13. Evaluation of *S. Typhimurium* reduction in *Hermetia illucens* following 2-hour prophylactic treatment with SPFM14 across increasing phage doses at 24 hours

Overnight cultures of SL1344 were washed and prepared as mentioned previously and serially diluted to an injection dose of 10^5 CFU in 10 μ L per larva. Phages (SPFM14) were prepared and diluted to yield 10^4 , 10^5 , and 10^6 PFU in 10 μ L per larva. Larvae were surface-rinsed in 70% ethanol, air-dried, anaesthetised on ice, and injected at the third-last metamere using a Hamilton syringe (10 μ L per injection). Experimental groups comprised an SL1344 control (10^5 , no phage), PBS, Phage only (10^5 , no bacteria) and no treatment groups, alongside three prophylactic (-2h) groups in which SPFM14 at 10^4 , 10^5 , and 10^6 PFU was administered 2 h before challenge with SL1344 10^5 CFU. Larvae were maintained at 28°C for 24 h before PFU/CFU counts.

For CFU enumeration, we followed the same protocol by dissecting whole guts into 500 μ L PBS, homogenised by vortexing, and 10 μ L of homogenate were spotted onto XLD agar in triplicate. Plates were incubated for ~16h at 37°C, and black H₂S-positive

colonies consistent with *Salmonella* were counted, and values were converted to CFU per larva. As background noise persisted on XLD, we continued using MALDI-TOF as a quality control step.

Phage titres (PFU per larva) were determined by a double-agar overlay using a solid LB base and a molten soft-agar overlay. Base agar was prepared at 10g LB + 4g agar per 400 mL H₂O; top (soft) agar was 5g LB + 1 g agar per 200 mL H₂O and held at 50-55°C. For each sample/dilution, 100 mL of gut homogenate was mixed with 200 mL of an exponentially growing phase culture of SL1344, combined with ~3 mL molten top agar, and poured over base-agar plates. After incubation of ~18-24 hours at 37°C, plaques were counted and PFU calculated with correction for plated volume and dilution.

5.14. Time-course of in vivo phage shedding following SPFM14 injection

Larvae were surface rinsed in 70% ethanol, anaesthetised on ice and injected at the third last metamere with 10 µL of SPFM14 prepared in PBS. Two input doses were tested, 10⁶ and 10⁷ PFU per larva (verified by plaque assay) and PBS-injected larvae, SPFM14 only and no-treatment groups served as controls. Larvae were maintained at 28°C and sampled at 2-, 4-, 6-, 8-, 28-, 48-, and 72-hours post-injection, n= 10 per dose per timepoint. Guts were extracted, and the same phage assay previously outlined allowed for counts to determine the rate of bacteriophage shedding in *Hermetia illucens*.

5.15. Results

5.16. Establishment and persistence of *S. enterica* serovar Typhimurium (SL1344) in BSFL

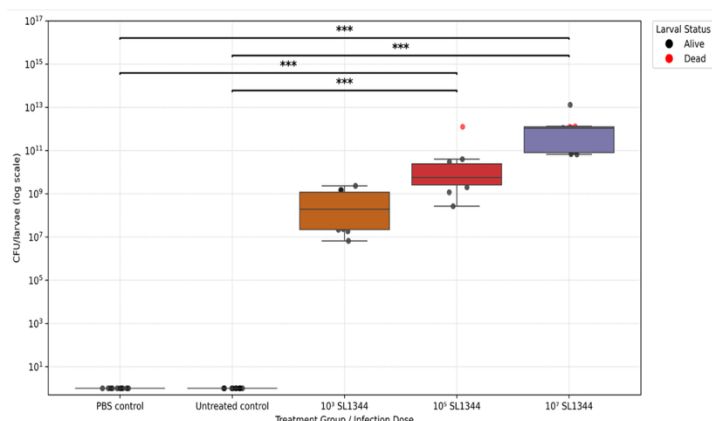
To establish a reproducible in vivo infection model for *Salmonella enterica* serovar Typhimurium (SL1344) in *Hermetia illucens*, larvae were challenged with increasing

bacterial doses and bacterial burden subsequently quantified (Figure 5.1A; Table 5.1). Recovery of SL1344 from larval gut homogenates demonstrated successful colonisation across all infection doses tested (10^3 , 10^5 10^7 CFU/larva) at 24 hours post-infection. A significant dose-dependent increase in bacterial burden was observed between treatment groups (Kruskal-Wallis $H = 46.36$, $p = 2.08 \times 10^{-9}$), confirming that increasing inoculation doses resulted in correspondingly greater levels of bacterial colonisation.

To determine whether colonisation could be maintained over time, larvae challenged with 10^5 CFU/larva were monitored at 24, 48 and 72 hours post-infection (Figure 5.1B; Table 5.2). SL1344 remained detectable throughout the experimental period, demonstrating that the bacterium could persist within the larval host for at least 72 hours following infection. Although a modest decline in median bacterial burden was observed over time, substantial bacterial loads remained recoverable at all sampling points, indicating prolonged colonisation rather than rapid clearance.

Importantly, mortality remained low throughout these experiments despite substantial bacterial burdens being recovered from infected larvae. Although some mortality was observed at higher infection doses (Table 5.1), most larvae survived infection and maintained detectable bacterial colonisation throughout the experimental period. These findings demonstrate that *H. illucens* larvae can tolerate substantial levels of SL1344 colonisation while maintaining survival, supporting the suitability of BSFL as a reproducible in vivo model for investigating host-pathogen interactions and evaluating bacteriophage-based intervention strategies.

2A



2B

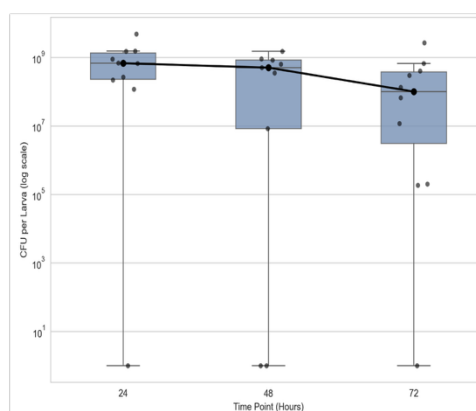


Figure 5.1 (A) Dose-dependent colonisation of SL1344 in BSFL across 10^3 , 10^5 , and 10^7 CFU/larva alongside PBS and Untreated controls ($n=10$ Kruskal-Wallis $H=46.36$, $p = 2.08 \times 10^{-9}$) (B) Time-dependent dynamics of *Salmonella enterica* serovar Typhimurium (SL1344) load in BSFL following infection of 10^5 CFU/larva at 24, 48, and 72 hours

Table 5.1 Summary of SL1344 colonisation in Black Soldier Fly Larvae (BSFL) following challenge with 103, 105, and 107 CFU/larva. Increasing inoculation doses resulted in progressively greater levels of SL1344 colonisation, while larval mortality remained low.

Infection dose (CFU/larva)	n	Alive	Dead	Mortality (%)	Median CFU/larva	Range (CFU/larva)
10^3	10	10	0	0	1.92×10^8	$6.65 \times 10^6 - 2.34 \times 10^9$
10^5	10	9	1	10	5.67×10^9	$2.67 \times 10^8 - 1.27 \times 10^{12}$
10^7	10	7	3	30	1.08×10^{12}	$6.67 \times 10^{10} - 1.30 \times 10^{13}$

Table 5.2 Persistence of *Salmonella enterica* serovar Typhimurium (SL1344) within Black Soldier Fly Larvae (BSFL) following infection with 105 CFU/larva over 24, 48 and 72 hours. SL1344 remained detectable throughout the experimental period, demonstrating sustained colonisation of the larval host with minimal associated mortality.

Time point (h)	n	Alive	Dead	Mortality (%)	Median CFU/larva	Range (CFU/larva)
24	10	10	0	0	6.75×10^8	0 - 4.84×10^9
48	10	9	1	10	5.68×10^8	0 - 2.04×10^{11}
72	10	10	0	0	1.00×10^8	0 - 2.67×10^9

5.17. Characterisation of XLD-grown gut microbiota and validation of *Salmonella* colonies by MALDI-TOF

To confirm the identity of bacterial colonies recovered from XLD agar following *Salmonella enterica* serovar Typhimurium (SL1344) challenge (Figure 5.2A-B), representative colonies were analysed using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS; Autobio MS1000) (Figure 5.3A-B). XLD agar was selected as a differential and selective medium for the recovery of presumptive *Salmonella* colonies; however, colony morphology alone was insufficient to reliably distinguish SL1344 from other gut-associated bacteria capable of growth under these conditions.

Visual inspection of XLD plates revealed considerable variation in colony morphology, including black-centred colonies typically associated with hydrogen sulphide production as well as yellow and pink colony morphotypes (Figure 5.2A-B). To verify colony identity, representative isolates from these morphotypes were subjected to MALDI-TOF analysis. Spectral profiles generated from individual colonies produced high-quality protein peak patterns suitable for species-level identification (Figure 5.3A).

MALDI-TOF analysis demonstrated that colonies recovered from XLD agar were not exclusively composed of *Salmonella*. Across the isolates analysed, four dominant bacterial taxa were identified: *Proteus mirabilis* (frequency = 28), *Providencia stuartii* (frequency = 25), *Morganella morganii* (frequency = 24), and *Klebsiella pneumoniae* (frequency = 22). In addition, colonies recovered from the positive-control SL1344 treatment were successfully identified as *Salmonella* spp., confirming that the challenge organism could be recovered and distinguished from background gut-associated bacteria.

These findings demonstrate that presumptive identification based solely on colony morphology would have resulted in misclassification of multiple isolates. The presence of several Enterobacteriaceae-associated species capable of growth on XLD agar highlights the importance of species-level validation when quantifying bacterial

recovery from BSFL gut homogenates. Consequently, MALDI-TOF analysis was used as a validation tool to confirm the identity of recovered colonies and inform interpretation of bacterial enumeration data throughout subsequent experiments.

Although MALDI-TOF analysis identified multiple culturable bacterial taxa, these data should not be interpreted as representative of total BSFL gut microbiome richness. Colonies were recovered using XLD agar, a selective medium designed to enrich for enteric bacteria, and therefore represent only a subset of culturable microorganisms capable of growth under these conditions. As such, the present dataset was used for colony validation rather than comprehensive microbiome profiling. Future studies employing culture-independent approaches, such as 16S rRNA gene amplicon sequencing or metagenomic sequencing, would be required to accurately characterise BSFL gut microbial diversity and species richness.

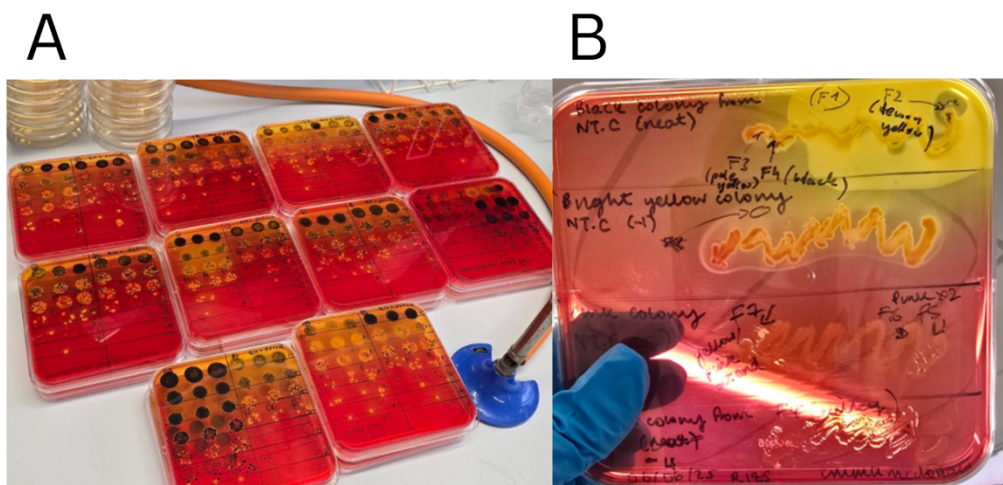


Figure 5.2 Identifying bacterial colonies on XLD in *Hermetia illucens* (A) XLD agar showing a mixture of colonies after injection of SPFM14 and *Salmonella Typhimurium* (SL1344). (B) colonies were picked and spread on XLD and LB agar (not shown) for MALDI-TOF analysis.

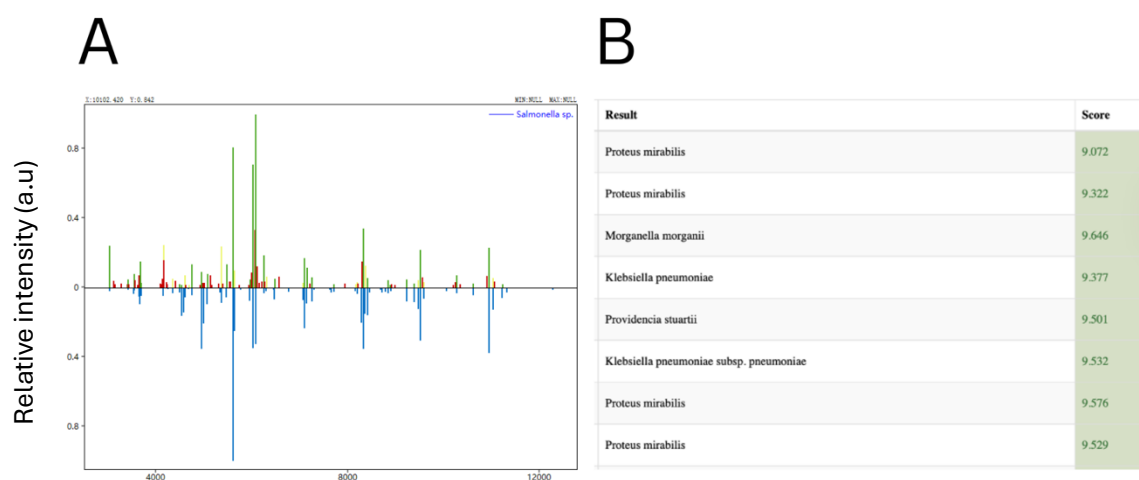


Figure 5.3 Identification of the most common bacterial species detected in the gut of *Hermetia illucens* from captured from XLD (A) Representative MALDI-TOF spectrum overlay for *Salmonella enterica* serovar Typhimurium (SL1344). The blue trace is the reference/library spectrum; coloured peaks are the spectrum acquired from a colony recovered from BSFL on XLD. (B) Top identifications (MALDI-TOF) of additional colonies recovered from XLD plates showed co-culturing of Enterobacterales commonly associated with insect microbiota, including *Proteus mirabilis*, *Morganella morganii*, *Klebsiella pneumoniae* (and subspecies), and *Providencia stuartii*.

5.18. Transmission Electron Microscopy of SPFM14

Negative-stain TEM revealed that SPFM14 possess isometric heads and long tails with a distinct baseplate and lateral fibres (Figure 5.4). Micrographs were acquired at ~50,000x following ammonium-acetate buffer exchange and 1% uranyl acetate staining. Particle dimensions were quantified in Fiji (ImageJ) from calibrated images: capsid diameter from the ellipse major axis fitted to the head (halo excluded) and tail length as a straight/segmented line from the head-tail junction to the end of the tail tube (lateral fibres excluded).

SPFM14 (n = 11), the capsid diameter was 128.3 ± 10.9 nm (median 128.8; range 104.2–142.3) and the tail (tube) length was 200.1 ± 12.5 nm (median 203.8; range 172.6–212.3). Representative particles are shown (Figure 5.4), where the annotated example measures ~115 nm for the capsid and ~211 nm for the tail, consistent with the cohort summary.

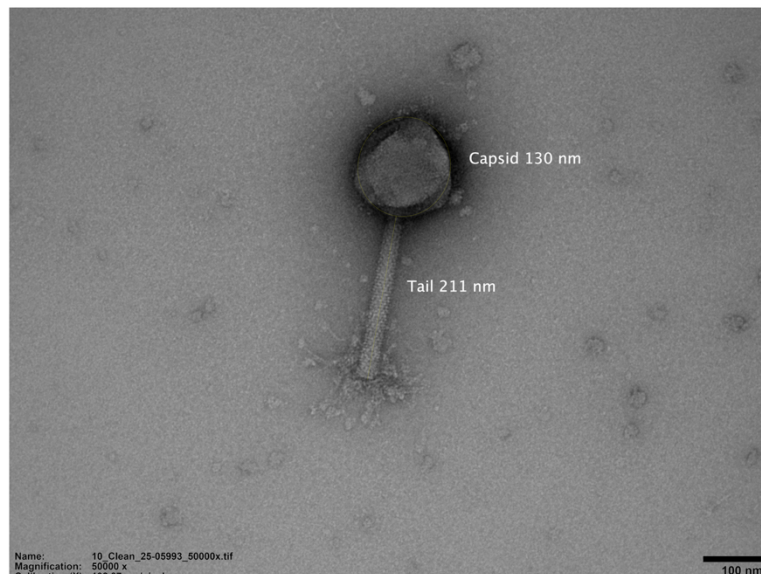


Figure 5.4 Negative-stain TEM of *Salmonella* bacteriophages SPFM14. Representative virions showing isometric heads and long tailed morphology; baseplate and lateral tail fibres are visible. Preparations were imaged at ~50,000 $\times$ following 0.1 M ammonium acetate buffer exchange and 1% uranyl acetate staining. Scale bars, 100 nm.

5.19. Experimental overview

To evaluate the efficacy of prophylactic bacteriophage treatment against *Salmonella enterica* serovar Typhimurium (SL1344) in *Hermetia illucens* larvae, a series of in vivo infection experiments were performed. Larvae were first administered SPFM14 at varying phage concentrations (10^4 , 10^5 , or 10^6 PFU/larva) prior to bacterial challenge with 10^5 CFU/larva SL1344. Following infection, larvae were maintained under controlled conditions and sampled at predetermined timepoints to assess bacterial colonisation dynamics.

At each sampling point, larvae were euthanised, homogenised, serially diluted, and plated on selective media to quantify viable *Salmonella* populations. Bacterial burden was expressed as colony forming units (CFU) per larva and compared across treatment groups. Control groups included untreated larvae, PBS-injected larvae, phage-only controls, and SL1344-only infected controls. A schematic overview of the experimental workflow is presented in Figure 5.5.

Methodology

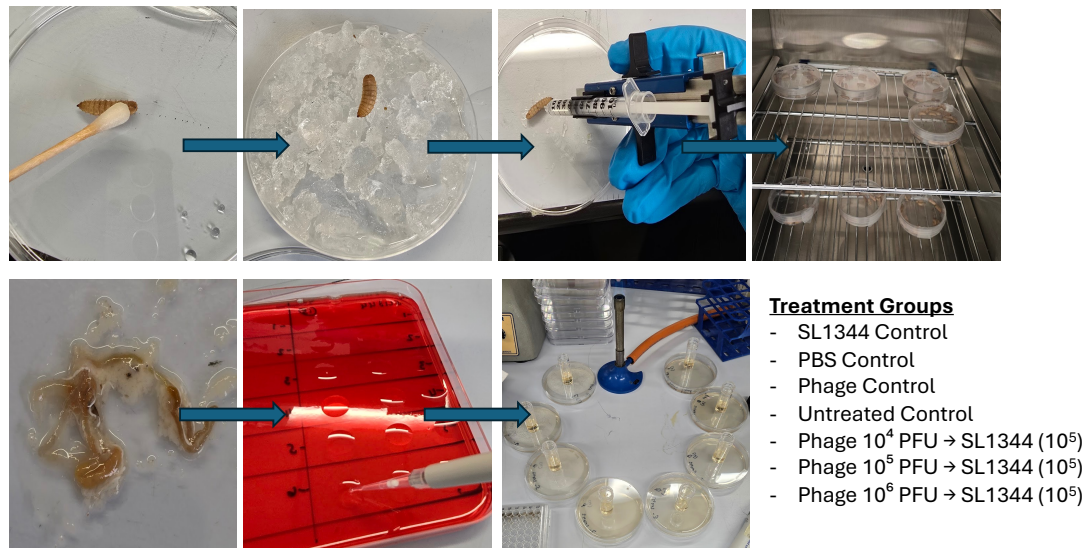


Figure 5.5 Experimental workflow for phage intervention in *Salmonella Typhimurium* (SL1344) infected *Black Soldier Fly Larvae*. Larvae were surface-sterilised with 70% ethanol, cold-anaesthetised on ice, and injected with bacteria (SL1344) and phage (SPFM14) according to the relevant treatment regimen (concurrent/prophylactic). Larvae were incubated for 24, 48, or 72 hours before gut dissection, homogenisation in PBS, serial dilution, and drop plating for CFU quantification. Phage titres (PFU/mL) were determined using standard double-layer agar assays.

5.20. Impact of concurrent SPFM14 administration on bacterial colonisation of *Salmonella Typhimurium* (SL1344) in *Hermetia illucens*

To investigate whether simultaneous administration of bacteriophage SPFM14 and *Salmonella enterica* serovar Typhimurium (SL1344) could reduce bacterial colonisation, larvae were challenged with equal doses of bacteria and phage (10^3 , 10^5 and 10^7 CFU/PFU per larva), and bacterial burden was quantified after 24 h (Figure 5.6). Analysis was performed using only surviving larvae to avoid mortality-associated bias in bacterial burden estimates.

A significant effect of infection dose on bacterial colonisation was observed (two-way ANOVA, $F_{2,52} = 46.85$, $p = 2.33 \times 10^{-12}$), with increasing inoculation doses resulting in progressively greater SL1344 burdens. Although no overall treatment effect was detected ($F_{2,52} = 0.0001$, $p = 0.992$), a significant interaction between infection dose and

treatment condition was identified ($F_{2,52} = 9.61$, $p = 2.80 \times 10^{-4}$), indicating that the effect of SPFM14 varied according to bacterial challenge dose.

Dose-matched comparisons revealed that concurrent SPFM14 treatment did not significantly reduce bacterial burden at 10^3 CFU/larva (Tukey post-hoc, $p = 0.0935$) or 10^5 CFU/larva ($p = 0.9868$) relative to the corresponding bacteria-only controls. However, at the highest challenge dose (10^7 CFU/larva), concurrent phage treatment resulted in a significant reduction in SL1344 burden ($p = 0.0157$), suggesting that SPFM14 exerted measurable antibacterial activity only under conditions of high bacterial density.

No surviving larvae exhibited complete bacterial clearance, and detectable SL1344 burdens were recovered from all individuals analysed. Consequently, the frequency of larvae with bacterial counts below the limit of detection was zero across all concurrent treatment groups.

Mortality remained low throughout the experiment (Table 5.3). Only two deaths were recorded across all treatment groups, corresponding to mortality rates of 10% within the 10^5 CFU/larva bacteria-only treatment and the 10^7 CFU/larva concurrent treatment. Fisher's exact tests identified no significant differences in mortality between bacteria-only and concurrent treatment groups at any infection dose (all $p = 1.000$), indicating that concurrent SPFM14 administration did not adversely affect larval survival.

Collectively, these findings demonstrate that concurrent administration of SPFM14 provided only limited reductions in SL1344 colonisation, with a significant effect observed only at the highest infection dose. Nevertheless, the consistently high bacterial burdens recovered from surviving larvae, coupled with the low mortality observed across treatments, further support the successful establishment of a robust and reproducible in vivo BSFL-SL1344 infection model capable of sustaining substantial *Salmonella* colonisation over time without imposing a major fitness cost on the larval host.

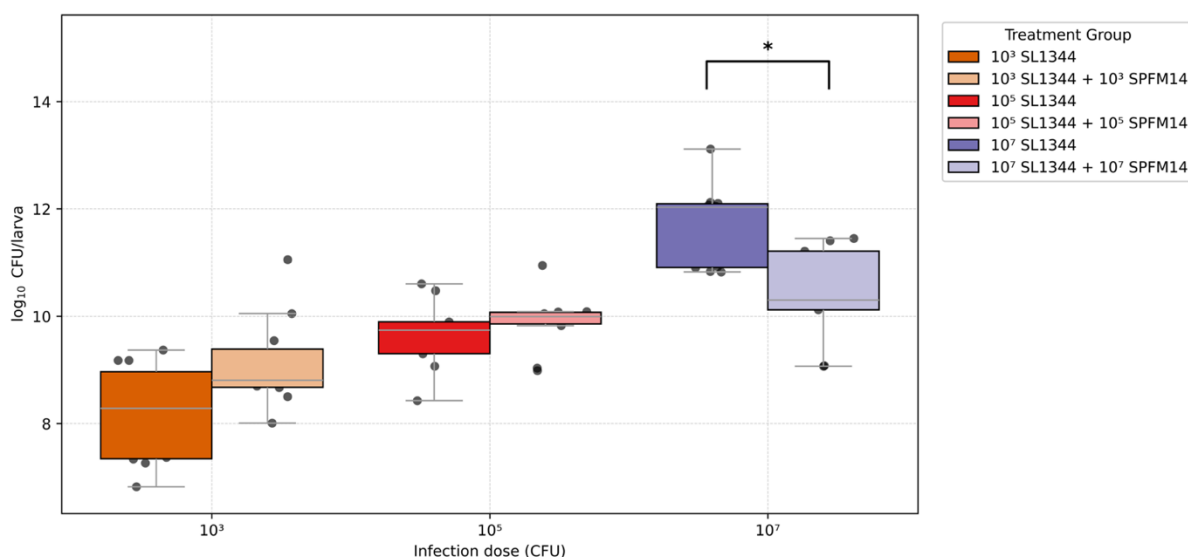


Figure 5.6 Impact of concurrent phage treatment on *Salmonella Typhimurium* (SL1344) colonisation in *Hermetia illucens* larvae. Larvae were inoculated with matched doses of *Salmonella Typhimurium* SL1344 (10^3 , 10^5 , or 10^7 CFU) either alone (controls) or concurrently with phage SPFM14 at equivalent titres (10^3 , 10^5 , or 10^7 PFU). Statistical analysis was performed using two-way ANOVA with Tukey's HSD post hoc test. $P < 0.05$.

Table 5.3 Summary of larval survival and mortality following concurrent SPFM14 and SL1344 challenge. Alive and dead are shown 24 h post-infection. No significant differences in mortality were observed between bacteria-only and concurrent phage-treated groups (Fisher's exact test, all $p = 1.000$).

Dose	Treatment	Alive	Dead	Mortality (%)
10^3	SL1344	10	0	0
10^3	Concurrent SPFM14 + SL1344	10	0	0
10^5	SL1344	9	1	10
10^5	Concurrent SPFM14 + SL1344	10	0	0
10^7	SL1344	10	0	0
10^7	Concurrent SPFM14 + SL1344	9	1	10

5.21. Effect of 1 h Prophylactic Phage Administration on *Salmonella enterica* serovar Typhimurium (SL1344) Colonisation and Survival in *Hermetia illucens*

To determine whether pre-exposure to bacteriophage SPFM14 could reduce subsequent *Salmonella* colonisation, larvae were treated with SPFM14 one hour before challenge with *Salmonella enterica* serovar Typhimurium (SL1344). Larvae were challenged with matched bacterial and phage doses (10^3 , 10^5 and 10^7 CFU/PFU per larva), and bacterial burden was quantified after 24 h (Figure 5.7). Analysis was

performed using surviving larvae only to avoid mortality-associated bias in bacterial burden estimates.

Dose-matched comparisons demonstrated that prophylactic SPFM14 administration had a dose-dependent impact on bacterial colonisation. No significant reduction in SL1344 burden was observed at the lowest challenge dose (10^3 CFU/larva; Mann-Whitney U test, Holm-adjusted $p = 0.870$). In contrast, prophylactic phage treatment significantly reduced bacterial burden at both 10^5 CFU/larva (Holm-adjusted $p = 0.0338$) and 10^7 CFU/larva (Holm-adjusted $p = 0.0051$) relative to the corresponding bacteria-only controls (Figure 5.7).

Within the bacteria-only groups, increasing inoculation dose resulted in progressively greater levels of colonisation (Kruskal-Wallis $H = 23.21$, $p = 9.12 \times 10^{-6}$), with significant differences observed between all dose comparisons following Holm-corrected Dunn's post hoc testing. A similar overall dose effect was observed within prophylactically treated larvae (Kruskal-Wallis $H = 17.38$, $p = 1.68 \times 10^{-4}$), although bacterial burdens at 10^3 and 10^5 CFU/larva were not significantly different following phage treatment, reflecting the reduction in colonisation achieved by SPFM14.

In contrast to the concurrent treatment experiment, complete bacterial clearance was observed in a subset of prophylactically treated larvae (Table 5.4). At both the 10^3 and 10^5 challenge doses, three surviving larvae exhibited bacterial burdens below the limit of detection following prophylactic SPFM14 administration. Consequently, only 6 of 9 surviving larvae retained detectable SL1344 within these treatment groups. No bacterial clearance events were observed in the corresponding bacteria-only controls, and all surviving larvae within these groups retained detectable *Salmonella* burdens. At the highest challenge dose (10^7 CFU/larva), all surviving larvae retained detectable SL1344 irrespective of treatment condition.

Mortality remained low throughout the experiment (Table 5.4). One death was recorded within both the 10^3 and 10^5 prophylactic treatment groups, while a single death was also observed within the 10^5 bacteria-only control group. No mortality was recorded within

the 10^7 treatment group. Fisher's exact tests comparing the proportion of larvae with detectable *Salmonella* revealed no statistically significant differences between treatment groups at any challenge dose (10^3 CFU/larva, $p = 0.0867$; 10^5 CFU/larva, $p = 0.2059$; 10^7 CFU/larva, $p = 1.000$). However, trends towards increased bacterial clearance were evident at the lower challenge doses following prophylactic phage administration.

Collectively, these findings demonstrate that prophylactic administration of SPFM14 was more effective than concurrent treatment, resulting in significant reductions in bacterial burden at intermediate and high infection doses and complete bacterial clearance in a subset of larvae. These results suggest that providing phages prior to bacterial exposure improves their ability to limit pathogen establishment within the larval host and supports further investigation of prophylactic phage-based intervention strategies in BSFL production systems.

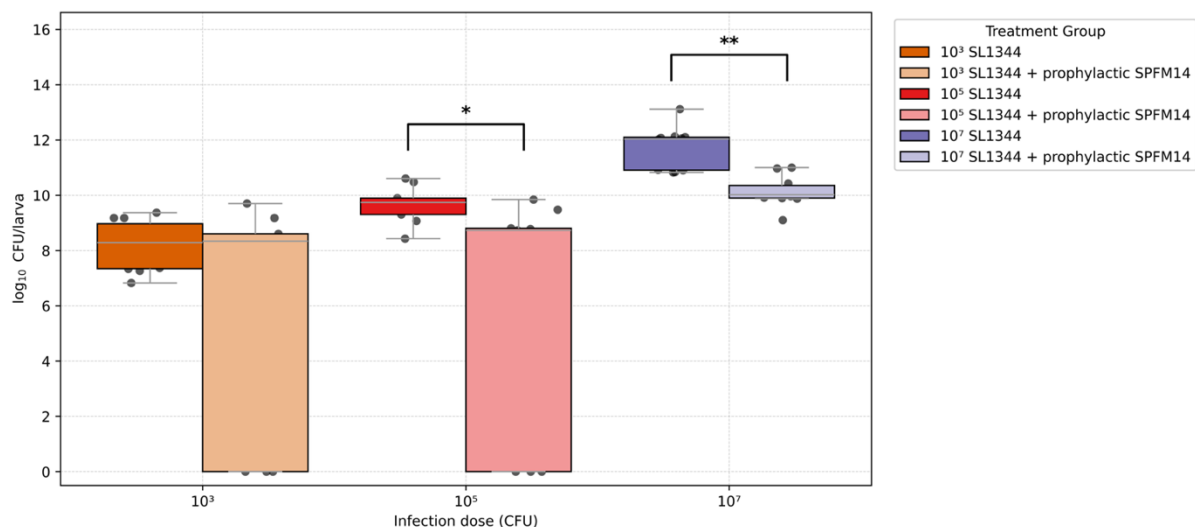


Figure 5.7 Dose-matched *Salmonella* (SL1344) burden at 24 h post-infection following 1 h prophylactic SPFM14 treatment in *Hermetia illucens* larvae. Larvae were pre-treated with phage SPFM14 1 h prior to challenge with *S. Typhimurium* SL1344 at infection doses of 10^3 , 10^5 , or 10^7 CFU ($n=10$ larvae per group). Statistical analysis was performed using Mann-Whitney U tests with Holm correction.

Table 5.4 Summary of larval survival, mortality, and detectable SL1344 recovery following (~1 h) prophylactic administration of SPFM14 prior to *Salmonella Typhimurium* (SL1344) challenge. Detectable SL1344 and below-detection frequencies were calculated using surviving larvae only. Complete bacterial clearance was observed in three larvae at both the 10^3 and 10^5 CFU challenge doses following prophylactic phage treatment.

Dose	Treatment	Alive	Dead	Mortality (%)	Detectable SL1344 (alive only)	Below detection (alive only)
10^3	SL1344	10	0	0	10/10	0/10
10^3	Prophylactic SPFM14	9	1	10	6/9	3/9
10^5	SL1344	9	1	10	9/9	0/9
10^5	Prophylactic SPFM14	9	1	10	6/9	3/9
10^7	SL1344	10	0	0	10/10	0/10
10^7	Prophylactic SPFM14	10	0	0	10/10	0/10

5.22. Comparison of concurrent and prophylactic SPFM14 treatment strategies

To directly compare the influence of treatment timing on phage efficacy, the outcomes of concurrent and prophylactic SPFM14 administration were evaluated across matched bacterial and phage doses (Table 5.5). Both treatment strategies produced reductions in SL1344 burden at the highest challenge dose (10^7 CFU/PFU per larva); however, prophylactic treatment consistently demonstrated greater efficacy than concurrent administration.

At 10^3 CFU/PFU per larva, neither treatment strategy produced a significant reduction in bacterial burden relative to bacteria-only controls. At 10^5 CFU/PFU per larva, concurrent treatment did not significantly affect colonisation, whereas prophylactic administration resulted in a significant reduction in bacterial burden and complete bacterial clearance in a subset of larvae. At 10^7 CFU/PFU per larva, both treatment strategies significantly reduced SL1344 colonisation, although the effect observed following prophylactic treatment was more pronounced.

Importantly, complete bacterial clearance was only observed following prophylactic administration, where three of nine surviving larvae exhibited bacterial burdens below

the limit of detection at both the 10^3 and 10^5 challenge doses. No clearance events were observed following concurrent treatment. Mortality remained low across all treatment groups and no treatment-associated increases in mortality were detected.

Collectively, these findings indicate that treatment timing influences phage efficacy within BSFL, with prophylactic administration providing greater protection against pathogen establishment than simultaneous phage-bacteria exposure.

Table 5.5 Comparison of concurrent and prophylactic SPFM14 administration against matched doses of *Salmonella enterica* serovar Typhimurium (SL1344) in *Hermetia illucens* larvae. Phages were administered at titres equivalent to the bacterial challenge dose (10^3 , 10^5 or 10^7 PFU per larva). Prophylactic treatment produced greater reductions in bacterial burden and was the only treatment strategy associated with complete bacterial clearance.

Bacterial challenge	Concurrent treatment outcome	Prophylactic treatment outcome
10^3 CFU SL1344 + 10^3 PFU SPFM14	No significant reduction in SL1344 burden ($p = 0.0935$)	No significant reduction in SL1344 burden ($p = 0.8698$)
10^5 CFU SL1344 + 10^5 PFU SPFM14	No significant reduction in SL1344 burden ($p = 0.9868$)	Significant reduction in SL1344 burden ($p = 0.0338$)
10^7 CFU SL1344 + 10^7 PFU SPFM14	Significant reduction in SL1344 burden ($p = 0.0157$)	Significant reduction in SL1344 burden ($p = 0.0051$)
Complete bacterial clearance	Not observed	Observed in 3/9 larvae at 10^3 CFU/PFU and 3/9 larvae at 10^5 CFU/PFU
Mortality	Low; no significant treatment effect	Low; no significant treatment effect

5.23. Effect of increasing prophylactic phage dose on *Salmonella* colonisation at 24 hours post-infection

Following the pilot studies described previously, a larger-scale prophylactic phage experiment was conducted to further investigate dose-dependent effects on *Salmonella* colonisation. In contrast to the pilot study ($n = 10$ larvae per treatment), the present experiment approximately doubled the sample size ($n = 20$ larvae per treatment) to improve statistical power. Additionally, the prophylactic treatment interval was increased from 1 hour to 2 hours prior to bacterial challenge to allow greater opportunity for phage establishment within the larval environment before exposure to *Salmonella Typhimurium* SL1344.

To determine whether prophylactic administration of bacteriophages reduced *Salmonella Typhimurium* SL1344 colonisation in BSFL, larvae were treated with increasing phage doses (10^4 , 10^5 and 10^6 PFU/larva) prior to infection with 10^5 CFU/larva SL1344. Bacterial loads were quantified 24 hours post-infection (Figure 5.8).

A significant overall effect of treatment was observed across the infected groups (Kruskal-Wallis $H = 20.10$, $p = 1.62 \times 10^{-4}$). Compared with the SL1344-only treatment, prophylactic phage administration resulted in significant reductions in bacterial burden at all tested phage doses. Mann-Whitney U tests with Holm correction demonstrated significant reductions following treatment with 10^4 PFU/larva (adjusted $p = 0.0020$), 10^5 PFU/larva (adjusted $p = 0.0061$), and 10^6 PFU/larva (adjusted $p = 1.24 \times 10^{-4}$).

The greatest reduction in bacterial burden was observed following administration of 10^6 PFU/larva, where median bacterial counts were approximately $1.7 \log_{10}$ CFU lower than the SL1344-only group. Despite this variability, median bacterial burdens decreased progressively as phage dose increased, supporting a dose-dependent prophylactic effect.

In addition to reductions in bacterial burden, increasing phage dose was associated with a greater number of larvae in which *Salmonella* could not be detected. No larvae in the SL1344-only group had bacterial loads below the limit of detection (0/17), whereas non-detectable bacterial counts were observed in 2/18 (11.1%), 4/18 (22.2%), and 7/17 (41.2%) larvae treated with 10^4 , 10^5 , and 10^6 PFU/larva, respectively. Mortality remained relatively low across all treatment groups, ranging from 0–15% (Table 5.6). Survival exceeded 85% in all infected treatments, indicating that substantial *Salmonella* colonisation could be established without causing extensive larval mortality. Prophylactic phage treatment did not significantly alter larval survival compared with the SL1344-only control, suggesting that the observed reductions in bacterial burden were not associated with changes in host survival over the 24-hour experimental period.

Fisher's exact testing demonstrated that only the highest prophylactic phage dose (10^6 PFU/larva) significantly increased the proportion of larvae with *Salmonella* loads below the limit of detection compared with the SL1344-only control ($p = 0.0072$), while lower phage doses did not significantly alter the proportion of detectable infections.

Together, these findings demonstrate a clear dose-dependent reduction in *Salmonella* colonisation following prophylactic phage administration. Increasing phage dose was associated with progressively lower bacterial burdens and a higher proportion of larvae with *Salmonella* loads below the limit of detection, with the 10^6 PFU treatment producing the strongest prophylactic effect.

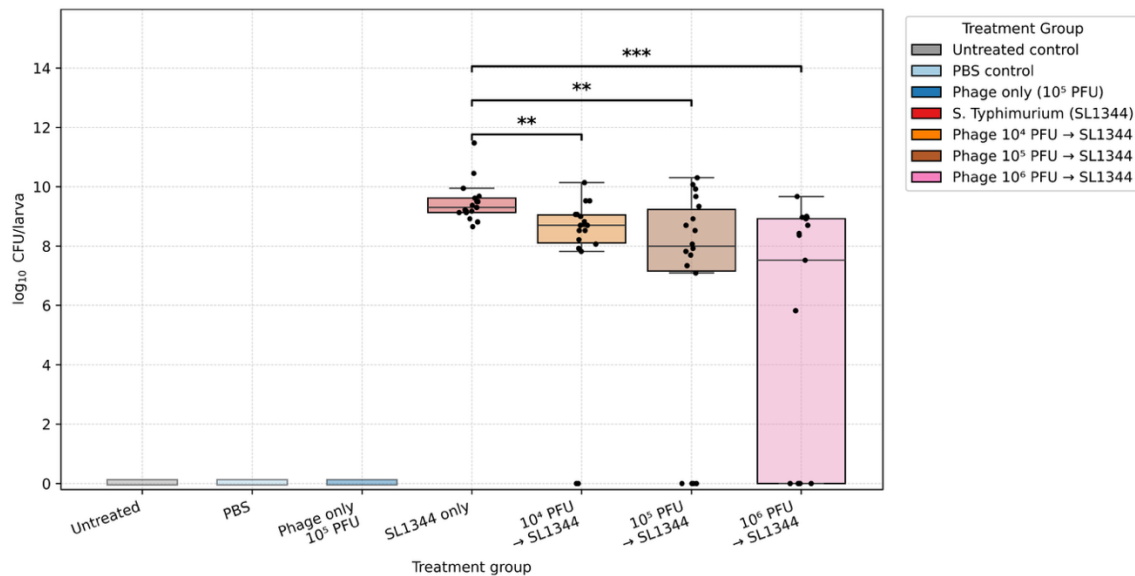


Figure 5.8 Dose-dependent reduction in *Salmonella Typhimurium* (SL1344) colonisation following prophylactic SPFM14 phage treatment in *Hermetia illucens* larvae at 24 h post-infection. Larvae ($n=20$) were injected with phage SPFM14 2 h prior (10^4 , 10^5 or 10^6 PFU) then infected with 10^5 CFU/larva SL1344. Statistical significance was assessed using Kruskal-Wallis with Dunn's post hoc test and Mann-Whitney U tests with Holm correction.

Table 5.6 Summary of larval survival, mortality and detectable *Salmonella Typhimurium* (SL1344) recovery following prophylactic SPFM14 administration at increasing phage doses.

Dose (PFU/larva)	Treatment	Alive	Dead	Mortality (%)	Detectable SL1344 (alive only)	Below detection (alive only)
-	Untreated	20	0	0	0/20	20/20
-	PBS	18	2	10	0/18	18/18
10 ⁵	Phage only	19	1	5	0/19	19/19
-	SL1344 only	17	3	15	17/17	0/17
10 ⁴	Prophylactic SPFM14	18	2	10	16/18	2/18
10 ⁵	Prophylactic SPFM14	18	2	10	14/18	4/18
10 ⁶	Prophylactic SPFM14	17	3	15	10/17	7/17

5.24. Time-dependent effects of prophylactic phage treatment on *Salmonella* colonisation

Following demonstration of a dose-dependent prophylactic effect at 24 hours post-infection, bacterial colonisation was monitored over a 72-hour period to determine whether reductions in *Salmonella Typhimurium* SL1344 burden were maintained over time. Larvae were pre-treated with SPFM14 at 10⁴, 10⁵, or 10⁶ PFU/larva two hours prior to infection with 10⁵ CFU/larva SL1344, and bacterial loads were quantified at 24, 48, and 72 hours post-infection (Figure 5.9A-C).

At 24 hours post-infection (Figure 5.9A), a significant overall treatment effect was observed among infected groups (Kruskal-Wallis H = 12.04, p = 0.0072). Compared with the SL1344-only control, only the highest phage dose (10⁶ PFU/larva) resulted in a significant reduction in bacterial burden (Holm-adjusted p = 0.0065), whereas reductions observed at 10⁴ and 10⁵ PFU/larva were not statistically significant.

At 48 hours post-infection (Figure 5.9B), a significant overall treatment effect remained evident (Kruskal-Wallis H = 9.59, p = 0.0224). Significant reductions in bacterial burden were observed following treatment with both 10⁵ PFU/larva (Holm-adjusted p = 0.0485) and 10⁶ PFU/larva (Holm-adjusted p = 0.0378), while the 10⁴ PFU treatment remained non-significant.

By 72 hours post-infection (Figure 5.9C), prophylactic phage treatment produced significant reductions in bacterial burden at all tested phage doses (Kruskal-Wallis $H = 15.19$, $p = 0.0017$). Mann-Whitney U tests with Holm correction demonstrated significant reductions for 10^4 , 10^5 , and 10^6 PFU/larva treatments (all adjusted $p = 0.0090$).

Median bacterial loads in the SL1344-only treatment decreased from approximately 6.75×10^8 CFU/larva at 24 hours to 1.00×10^8 CFU/larva at 72 hours. Despite this decline, prophylactic phage treatment consistently resulted in lower bacterial burdens than the infected control throughout the experimental period. Importantly, reductions in bacterial burden were maintained despite the persistence of detectable infection within the untreated SL1344 group at all sampling timepoints. Increasing phage dose was generally associated with lower median bacterial loads and greater numbers of larvae with bacterial counts below the limit of detection, particularly during the early stages of infection.

Analysis of detectable versus non-detectable infections further supported the efficacy of prophylactic phage treatment (Table 5.7). At 24 hours, only the highest phage dose significantly increased the proportion of larvae with bacterial loads below the limit of detection compared with the SL1344-only treatment (Fisher's exact $p = 0.0128$). No significant differences were observed at 48 hours. By 72 hours post-infection, all prophylactic phage doses significantly increased the proportion of larvae with bacterial loads below the limit of detection relative to the SL1344-only treatment (10^4 PFU, $p = 0.0198$; 10^5 PFU, $p = 0.0198$; 10^6 PFU, $p = 0.0128$).

Together, these findings demonstrate that prophylactic phage treatment reduced *Salmonella* colonisation throughout the 72-hour experimental period. While the strongest reductions were initially observed at the highest phage dose, significant decreases in bacterial burden became evident across all prophylactic treatments by 72 hours post-infection, indicating a sustained and time-dependent prophylactic effect.

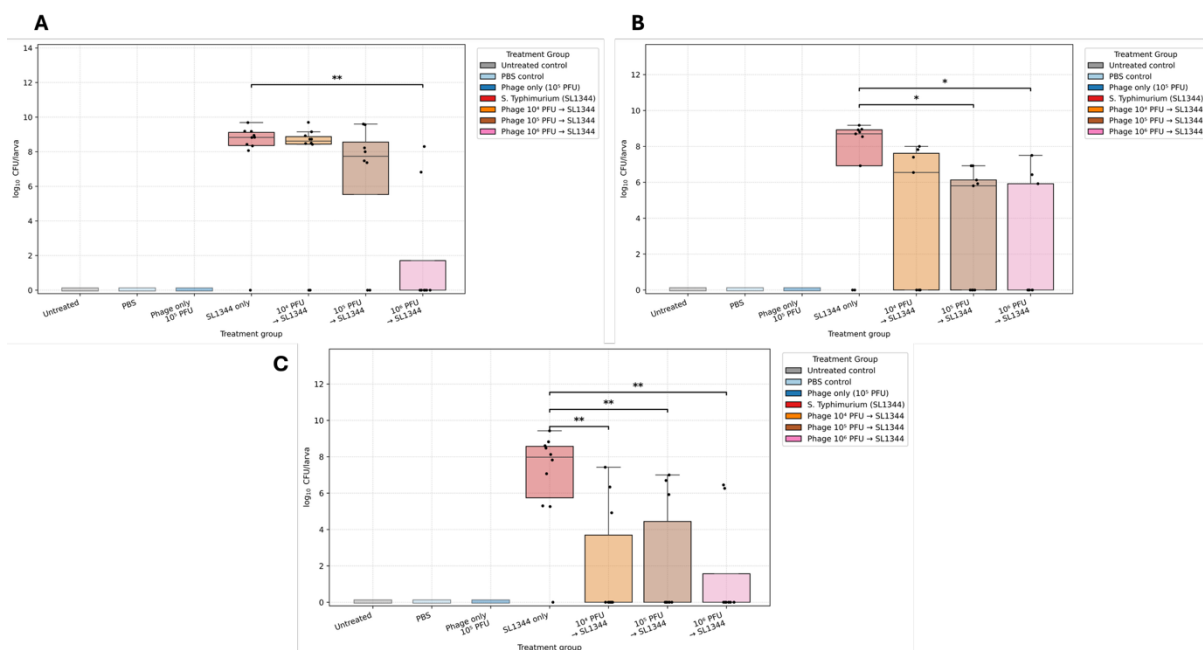


Figure 5.9 *Salmonella Typhimurium* (SL1344) gut colonisation in alive *Hermetia illucens* larvae following prophylactic phage treatment at 24 h (A), 48 h (B), 72 h (C) (n = 10). Larvae were pre-treated with 10⁴, 10⁵ or 10⁶ PFU of SPFM14 phage prior to SL1344 challenge. Statistical significance was assessed using Kruskal-Wallis tests with Dunn's post hoc comparisons and Mann-Whitney U tests with Holm correction.

Table 5.7 Survival, mortality and detectable *Salmonella Typhimurium* (SL1344) colonisation in BSFL following prophylactic SPFM14 treatment over a 72-hour infection period.

Time (h)	Dose (PFU/larva)	Treatment	Alive	Dead	Mortality (%)	Detectable SL1344 (alive only)	Below detection (alive only)
24	-	Untreated	10	0	0	0/10	10/10
24	-	PBS	10	0	0	0/10	10/10
24	10 ⁵	Phage only	10	0	0	0/10	10/10
24	-	SL1344 only	10	0	0	9/10	1/10
24	10 ⁴	Prophylactic SPFM14	10	0	0	8/10	2/10
24	10 ⁵	Prophylactic SPFM14	8	2	20	6/8	2/8
24	10 ⁶	Prophylactic SPFM14	8	2	20	2/8	6/8
48	-	Untreated	10	0	0	0/10	10/10
48	-	PBS	10	0	0	0/10	10/10
48	10 ⁵	Phage only	10	0	0	0/10	10/10
48	-	SL1344 only	9	1	10	7/9	2/9
48	10 ⁴	Prophylactic SPFM14	7	3	30	4/7	3/7
48	10 ⁵	Prophylactic SPFM14	9	1	10	5/9	4/9

48	10 ⁶	Prophylactic SPFM14	9	1	10	3/9	6/9
72	-	Untreated	10	0	0	0/10	10/10
72	-	PBS	9	1	10	0/9	9/9
72	10 ⁵	Phage only	10	0	0	0/10	10/10
72	-	SL1344 only	10	0	0	9/10	1/10
72	10 ⁴	Prophylactic SPFM14	10	0	0	3/10	7/10
72	10 ⁵	Prophylactic SPFM14	10	0	0	3/10	7/10
72	10 ⁶	Prophylactic SPFM14	8	2	20	2/8	6/8

5.25. Rate of bacteriophage SPFM14 shedding from the *Hermetia illucens* larval gut

In preliminary trials, routine sampling failed to recover viable phage from larvae, suggesting that our sampling times may have missed the shedding window. We therefore undertook a time-dependent assay to quantify the shedding dynamics of the SPFM14 phage following injection, to define when titres are detectable and how rapidly they decline.

We looked to determine how quickly *Hermetia illucens* shed the bacteriophage SPFM14 (10⁶ and 10⁷ PFU/ml) over 2, 4, 6, 8, 24, 48, and 72 hours. Across both doses, shedding was highest in the first 2-4h and then fell by ~2-3 orders of magnitude by 6-8h, with sporadic low positives thereafter (Figure 5.14). At 10⁷ PFU/larva, shedding was detected in 100% of larvae at 2-8h and remained occasionally detectable at 24-48h (20% positive) but was undetectable by 72h. Medians (PFU per larva) at 10⁷ were 2.75 x 10⁵ (2h), 5.52 x 10⁴ (4h), 4.63 x 10² (6h), and 6.83 x 10³ (8h). At 10⁶ PFU/larva, detection rates were 60% (2 h), 90% (4 h), 100% (6h), 50% (8h), dropping to 10% at 24-48h and 0% at 72h; medians were 1.03 x 10⁵ (2h), 1.36 x 10⁴ (4h), 3.73 x 10³ (6h), and 1.85 x 10² (8h). Thus, the higher inoculum generally yielded greater shedding early (10⁷ vs 10⁶ medians were ~2.7x at 2h and ~4.1x at 4h), while by 6-8h both doses had declined sharply, with distributions overlapping and only rare low-level shedding beyond 24 h. Overall,

shedding is transient and front-loaded, largely confined to the first 8h post-injection, with dose-dependent magnitude early and near-complete clearance by 72h.

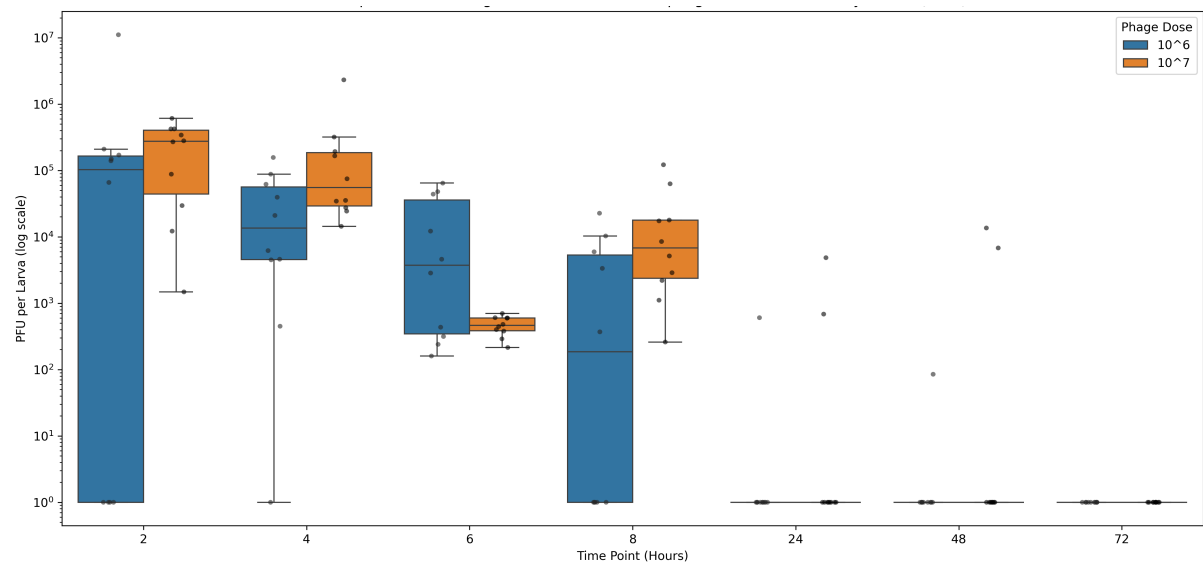


Figure 5.10 Time-dependent shedding of SPFM14 from *Hermetia illucens* following injection at 10^6 (blue) or 10^7 (orange) PFU. Points are individual larvae; boxes show IQR/median on a log₁₀ scale. Shedding peaks by 2–4 h, declines sharply by 6–8 h, and is largely undetectable by 24 h at both doses.

5.26. Limitations in interpreting phage replication and host-phage dynamics

Although significant reductions in *Salmonella Typhimurium* burden were observed following prophylactic administration of SPFM14, caution is required when interpreting the mechanisms responsible for this effect. The present study demonstrated that phage treatment was associated with reduced bacterial loads within the gut of *Hermetia illucens*, particularly at higher prophylactic doses; however, the available data do not provide direct evidence of sustained in vivo phage replication within the larval host. While viable phage was recovered from the gut during the first few hours following administration, phage abundance and bacterial burden were not quantified simultaneously throughout infection. Consequently, it is not possible to directly determine whether reductions in bacterial density were associated with active amplification of phage populations within the gut, transient lytic activity shortly after administration, or a combination of phage-mediated and host-mediated effects.

The interpretation of phage efficacy within animal hosts is inherently complex due to the dynamic nature of phage-host interactions. Successful phage replication depends upon numerous biological and environmental factors, including bacterial density, adsorption efficiency, latent period, burst size, spatial distribution of susceptible host cells, and persistence of phage particles within the host environment. Previous studies have demonstrated that SPFM14 possesses strong lytic activity against *Salmonella* spp. and can significantly reduce intestinal colonisation in poultry models (Thanki, et al., 2023). Therefore, the rapid decline in recoverable phage observed within *Hermetia illucens* is unlikely to reflect an inherent inability of SPFM14 to infect susceptible *Salmonella* hosts. Instead, it may indicate that phage-host interaction dynamics within the BSFL gut differ from those observed in previously characterised vertebrate systems, or that sampling the gut alone is missing other compartments where phage persist.

Consequently, although the findings support a prophylactic effect of SPFM14 against *Salmonella Typhimurium* within *Hermetia illucens*, they should not be interpreted as definitive evidence of sustained phage amplification within the larval host.

Future studies incorporating synchronous PFU and CFU measurements across multiple time points would allow direct assessment of phage-host interaction kinetics within *Hermetia illucens* and help determine whether reductions in *Salmonella* burden are associated with active phage amplification, transient lytic activity, host-mediated clearance, or a combination of these processes.

5.27. Discussion

This study set out to determine whether SPFM14 can be positioned for prophylactic control of *Salmonella Typhimurium* (SL1344) in Black Soldier Fly Larvae (BSFL; *Hermetia illucens*) and to determine the time window of phage persistence/activity in the larval gut.

Salmonella enterica serovar Typhimurium is a leading cause of food-borne disease globally, across high- and low-income settings (Mayo Clinic, 2025) (Popa & Papa, 2021). Most non-typhoidal *Salmonella* (NTS) infections are foodborne, linked to contaminated poultry and meat, eggs, fresh produce, and even processed items (e.g., nut butters, flour) (CDC, 2024). The global burden remains substantial: recent WHO/IHME estimates indicate ~73.9 million NTS diarrhoeal cases and ~61,600 deaths in 2019, while the invasive NTS (iNTS) burden is concentrated in sub-Saharan Africa (GBD 2021 ~510,000 incident cases (He, et al., 2025)), disproportionately affecting young children and immunocompromised adults (World Health Organization, 2018) (Morpeth, Ramadhani, & Crump, 2009). Concerningly, the WHO GLASS 2025 summary signals rising fluoroquinolone resistance in NTS, in 2023, ciprofloxacin non-susceptibility reached ~18% in bloodstream and ~16% in gastrointestinal infections in 2023 (with Europe notably higher ~36% and ~24%, respectively) (EFSA and ECDC, 2024) (WHO, 2025). These trends strengthen the rationale for alternative therapies, including targeted bacteriophage.

Bacteriophages offer a mechanistically precise, evolutionarily adaptable countermeasure to *Salmonella* in healthcare, feed/food chains and animal production systems. These bacterial-eating viruses attach to strain-specific surface receptors, inject their genomes, and, in the lytic cycle, redirect host transcription and metabolism to produce progeny virions before lysing the cell. This combination of strict host specificity and obligate bactericidal activity distinguishes phages from broad-spectrum antibiotics. Due to this, it makes them attractive where selective removal of a target pathogen is required while preserving commensals (Rai & Kumar, 2022). Importantly, phages co-evolve with their hosts, offering a dynamic countermeasure as bacterial

populations change, and they can be deployed as cocktails to broaden coverage and suppress resistance emergence (Koskella & Brockhurst, 2014) (Abedon, Danis-Wlodarczyk, & Wozniak, 2021) (Kim, et al., 2024). In food and agriculture, phages have crossed from concept to practice: in the United States, several preparations are cleared for food safety applications (e.g., *Listeria*-targeting additives [21 CFR § 172.785] and *E. coli* O157:H7 processing aids), establishing real regulatory pathways for targeted, non-antibiotic interventions (National Archives, 2025) (Vikram, Callahan, Woolston, Sharma, & Sulakvelidze, 2022) (Vikram, Tokman, Woolston, & Sulakvelidze, 2020).

Our choice of SPFM14 is grounded in prior work by Thanki et al. characterising a family of Seoulvirus-like jumbo myophages. These phages exhibited broad activity against multi-drug resistant (MDR) livestock-associated *Salmonella*, and notably, SPFM14 were among strains that lysed 100% of a diverse UK panel including *Salmonella* Typhimurium, *Salmonella* 4,12:i:-, *Salmonella* 4,5,12:i:-, *Salmonella* bovismorbificans and *Salmonella* Derby (Thanki, Brown, Millard, & Clokie, 2019). Building on this genomic foundation, the same group showed that dried, feed-delivered phage cocktails reduce gut colonisation in pigs and, separately, that an SPFM cocktails decrease *Salmonella* carriage in broiler chickens with favourable performance metrics (Thanki, et al., 2022) (Thanki, et al., 2022) (Thanki, et al., 2023). These studies validate prophylactic, pre-exposure delivery of SPFM phages in food-production systems and provide a direct precedent for our preventative strategy in BSFL.

Before the efficacy of phage therapy could be evaluated, it was first necessary to establish a reliable and reproducible *Salmonella* challenge model within *Hermetia illucens*. A major outcome of this chapter was the successful development of such a model, demonstrating that *Salmonella* Typhimurium SL1344 can consistently colonise the larval gut while producing minimal mortality. Across the challenge experiments, bacterial burden remained quantifiable over extended time periods, allowing the progression of infection and the impact of intervention strategies to be monitored. Importantly, this provides a robust platform for future investigations of pathogen-host interactions and disease control within BSFL production systems.

The establishment of a disease challenge model represents a significant advance for Black Soldier Fly research. While BSFL are increasingly used in waste bioconversion, animal feed production and circular economy applications, there remains a limited number of experimentally validated infection models available for studying pathogen colonisation and intervention strategies. The ability to reproducibly introduce, recover and quantify *Salmonella* from the larval gut creates opportunities to investigate pathogen persistence, microbial competition, host immune responses and novel biocontrol approaches under controlled conditions. Furthermore, the low mortality observed throughout the challenge period suggests that *Hermetia illucens* can act as a practical infection model in which changes in pathogen burden can be assessed without the confounding effects of extensive host mortality. Consequently, the challenge model developed in this study provides an important experimental framework for future studies examining bacterial pathogens, phage therapy and host-pathogen interactions in BSFL.

In addition to establishing a reproducible *Salmonella* challenge model, this study utilised MALDI-TOF mass spectrometry to verify the identity of colonies recovered from XLD agar and assess the extent to which non-target bacterial species contributed to colony counts. Although XLD agar is widely used as a selective medium for *Salmonella* isolation, several members of the Enterobacteriaceae can produce colony morphologies that resemble *Salmonella*, particularly within complex biological samples. The use of MALDI-TOF therefore provided an important validation step, confirming the presence of *Salmonella Typhimurium* while simultaneously identifying a range of background bacterial species including *Proteus*, *Providencia*, *Morganella* and *Klebsiella* spp.

These findings highlight the complexity of the BSFL gut microbiota and demonstrate that selective media alone may overestimate pathogen abundance when colony identity is inferred solely from morphology. Consequently, the incorporation of MALDI-TOF strengthened confidence in the bacterial burden data presented throughout this chapter by confirming that colonies classified as *Salmonella* were genuinely representative of the target pathogen.

It is important to note that the MALDI-TOF analysis performed in this study was designed as a targeted validation approach rather than a comprehensive microbiome characterisation exercise. The primary objective was to confirm colony identity and identify potential confounding species recovered on XLD agar, rather than to quantify community diversity, richness or abundance. As a result, analyses typically associated with microbiome studies, such as rarefaction curves, diversity metrics and community structure modelling, were beyond the scope of the experimental design. The dataset generated was therefore not intended to provide a complete representation of the BSFL gut microbiome, but rather to validate the microbiological methods used throughout the challenge model and phage efficacy experiments. Future studies incorporating high-throughput sequencing approaches, including 16S rRNA gene sequencing or metagenomics, would allow more detailed investigation of microbial community dynamics during *Salmonella* colonisation and phage treatment.

Negative-stain TEM (Figure 5.4) showed that SPFM14 have isometric heads attached to long, contractile tails with a discernible baseplate and lateral fibres, the hallmarks of myoviruses within the class *Caudoviricetes*, and as described by Thanki and colleagues (Thanki, Brown, Millard, & Clokie, 2019). The large heads we measured, and long tails, are consistent with packaging demands of >200kb genomes and with myoviral adsorption/injection machinery that favours rapid host engagement, mechanistic features desirable for prophylaxis therapy.

The successful establishment of a reproducible *Salmonella* challenge model enabled the evaluation of prophylactic and concurrent phage intervention strategies within *Hermetia illucens*. Collectively, the findings demonstrate that SPFM14 can reduce *Salmonella Typhimurium* colonisation within the larval gut, although the magnitude of this effect was strongly influenced by treatment timing, phage dose and the duration of infection.

Concurrent administration of SPFM14 and *Salmonella Typhimurium* produced only limited reductions in bacterial colonisation. Significant decreases in bacterial burden were observed only at the highest challenge dose (10^7 CFU/PFU per larva), while no significant effects were detected at the lower challenge doses. These findings suggest

that simultaneous exposure of phage and pathogen may provide insufficient opportunity for effective suppression before bacterial colonisation becomes established. Successful phage therapy depends upon encounters between susceptible bacterial hosts and infective phage particles, and the limited efficacy observed during concurrent treatment may reflect constraints on these early interactions within the larval environment. Despite the limited impact on bacterial burden, the ability to recover substantial *Salmonella* loads across all challenge doses further demonstrates the robustness of the infection model and confirms that *Hermetia illucens* can sustain significant *Salmonella* colonisation with minimal associated mortality.

In contrast, prophylactic administration consistently outperformed concurrent treatment. Providing SPFM14 prior to bacterial challenge resulted in significant reductions in bacterial burden at both intermediate and high challenge doses, while complete bacterial clearance was observed in a subset of larvae at the lower infection doses. These findings indicate that treatment timing plays a critical role in determining phage efficacy and suggest that establishing a phage population within the host before pathogen exposure may improve the likelihood of successful bacterial control. Similar observations have been reported in livestock-associated phage therapy studies, where preventative administration often provides greater protection than post-exposure treatment due to the ability of phages to encounter and infect susceptible bacteria immediately following pathogen introduction (Thanki, et al., 2022) (Thanki, et al., 2023). The present findings therefore support the use of prophylactic phage strategies as a potentially practical approach for pathogen management within BSFL production systems.

The larger-scale prophylactic experiment further demonstrated a clear dose-dependent relationship between phage administration and bacterial suppression. Increasing SPFM14 dose resulted in progressively lower *Salmonella* burdens and increased frequencies of larvae in which bacterial counts fell below the limit of detection. Significant reductions in bacterial burden were observed at all tested phage doses, while the highest dose (10^6 PFU per larva) produced the greatest reduction in colonisation and the highest frequency of apparent bacterial clearance. These findings

are consistent with established principles of phage therapy, whereby increasing phage-to-host ratios increase the probability of successful host infection and bacterial lysis. Importantly, the observed reductions occurred without substantial impacts on larval survival, indicating that the intervention was well tolerated by the host and supporting the suitability of bacteriophage-based approaches for BSFL-associated pathogen control.

Monitoring bacterial colonisation over a 72-hour period revealed that the prophylactic effect was maintained throughout the experimental timeframe. While the strongest reductions were initially observed at the highest phage dose, significant decreases in bacterial burden became evident across all prophylactic treatment groups by 72 hours post-infection. Interestingly, the relative impact of treatment became more apparent over time despite an overall decline in *Salmonella* burden within the untreated control group. This observation suggests that early reductions in pathogen establishment may alter subsequent colonisation dynamics within the larval gut, resulting in long-term differences in bacterial burden between treated and untreated larvae. Furthermore, the increasing proportion of larvae exhibiting bacterial counts below the limit of detection at later time points indicates that prophylactic phage treatment may influence both the magnitude and persistence of *Salmonella* colonisation.

The collective findings presented in this chapter are particularly notable when considered alongside the broader literature describing phage-mediated control of *Salmonella*. Previous studies have demonstrated that SPFM14 possesses broad lytic activity against livestock-associated *Salmonella* strains and can reduce intestinal colonisation within poultry production systems (Thanki, Brown, Millard, & Clokie, 2019) (Thanki, et al., 2023). The present work extends these observations to an entirely different host system and demonstrates, for the first time, that prophylactic phage treatment can significantly reduce *Salmonella* colonisation within Black Soldier Fly larvae. Given the increasing industrial ability to reduce pathogen burden without reliance on conventional antimicrobials represents a potentially valuable biocontrol strategy.

An important consideration for the long-term application of bacteriophage therapy is the potential emergence of phage-resistant bacterial populations. Bacteria can evolve resistance through multiple mechanisms including receptor modification, receptor masking, restriction-modification systems and CRISPR-Cas mediated defence pathways. Although resistance development was not assessed within the present study, the relatively short experimental timeframe may have limited opportunities for resistant populations to emerge and become dominant. Although the present study ultimately utilised SPFM14, future commercial applications may benefit from incorporating multiple phages within a cocktail formulation to minimise resistance development and broaden host coverage (Abedon, Danis-Włodarczyk, & Wozniak, 2021). Future studies should therefore investigate whether prolonged exposure of *Salmonella* within BSFL production systems results in the emergence of phage-resistant variants and determine whether additional phages should be incorporated into therapeutic cocktails to maintain efficacy over extended periods.

In parallel, plaque assays performed across all the above time points did not recover detectable phage, despite strong bacterial suppression. This led us to believe there was rapid shedding from the gut of *Hermetia illucens*, which put in motion our next observation to quantify phage kinetics in the larval gut over 2, 4, 6, 8, 12, 24, 48, and 72 hours.

Phage shedding was rapid and transient, with peak PFU occurring within the first 2-4 hours post-injection at both doses tested. Shedding declined sharply at 6-8 hours with only sporadic low levels after 24 hours, and a complete loss by 72 hours. In terms of SPFM14, it appears they don't persist for long periods of time within the gut of *Hermetia illucens*.

Paired CFU-PFU data in insects are scarce, with few models reporting gut phage kinetics alongside bacterial burden (Table 5.4). In *Musca domestica*, intestinal PFU remain detectable for ≥ 4 days under continuous oral dosing every 24 h, and in other studies, *Galleria mellonella* report detection up to 72h. Interestingly, *Hermetia illucens* appears to shed bacteriophage SPFM14 much quicker than other models; however, this

may appear different when continuous or repeated oral dosing occurs rather than a single bolus. Additionally, this study didn't quantify PFU within the haemolymph, which can exhibit different persistence than the gut.

Table 5.8 Summary of insect infection models reporting bacterial burden outcomes and phage persistence windows.

Model/Host	Pathogen/Target	Phage approach	Regimen	Bacterial outcome	Phage detection window	Source
<i>Galleria mellonella</i>	<i>Salmonella</i> spp	(ST-W77, SE-W109, SPFM17)	Prophylaxis : 2h pre-challenge	Undetectable by 24 h	Phage still detected in larva at 72h	(Nale, et al., 2021)
<i>Galleria mellonella</i>	MRSA infection (biofilm on K-wires)	Sb-1	Prophylaxis : 1h pre-challenge	Sb-1 prevented colonisation on K-wires	Detected up to 24 h in haemolymph	(Materazzi, et al., 2022)
<i>Galleria mellonella</i>	<i>B. cenocepacia</i> strains K56-2 and C6433	KS4, KS4-M, DC1, KS12	Phage given immediately after infection and delayed intervals	Survival scored only	no decrease in KS4-M alone group at 48h KS12 or KS14 alone show gradual decline over 48hrs	(Seed & Dennis, 2009)
<i>Musca Domestica</i>	<i>Pseudomonas aeruginosa</i>	Y12Pw	Oral Feeding (wheat bran 10 ⁸ PFU/ml)	≈90% reduction at 24 h	Phage still detected in intestine on day 4	(Zhang, et al., 2021)

In the case of *Hermetia illucens*, the data is most consistent with an initial boost from the phage in infected *Salmonella Typhimurium* larvae. While phage is abundant in the

first few hours (2-8h) within the larval gut, it appears to offer early reduction via the phage's lytic cycle capabilities, buying time for the larval immune system to contain the rest of the infection.

The potential contribution of the larval immune system to bacterial clearance should also be considered. Previous chapters demonstrated that *Hermetia illucens* possesses a diverse repertoire of antimicrobial peptides (AMPs), including defensin, cecropin and attacin family members, alongside pathogen recognition receptors such as TLR6. These immune effectors are known to contribute to antibacterial defence and may influence the outcome of *Salmonella* infections. Consequently, the reductions in bacterial burden observed following prophylactic phage treatment may not be attributable solely to direct phage-mediated lysis. Early reductions in bacterial density could alter the host-pathogen interaction trajectory, reducing pathogen loads to levels that are more readily controlled by endogenous immune responses. It is therefore possible that the prophylactic effect observed in this study reflects a combination of phage-mediated bacterial killing and host immune activity. However, antimicrobial peptide expression and immune pathway activation were not quantified during infection, preventing direct assessment of their contribution to bacterial clearance. Future studies should therefore investigate AMP expression and immune signalling dynamics alongside phage treatment to better understand the interaction between bacteriophage therapy and innate immunity in *Hermetia illucens*, which could be further supported by in situ hybridisation and RNAScope. It could also expand on phage delivery, i.e., (i) compare single-dose vs micro-dosed feeding in BSFL to test whether sustained delivery prolongs gut PFU, (ii) compare different phage types to assess stability, and (iii) include matched gut and haemolymph sampling to resolve compartment-specific kinetics from CFU/PFU, and to determine if phage modulates innate immune markers/alters microbiome. To our knowledge, this is the first work in Black Soldier Fly larvae to deliver a proof-of-concept for phage therapy, defining a short but impactful prophylactic window.

Although the present study focused primarily on bacteriophage-mediated intervention, the robust and sustained *Salmonella* colonisation model established here provides an

important opportunity for future studies investigating the contribution of BSFL innate immunity to infection control. Future work could quantify the temporal expression of antimicrobial peptides and pattern-recognition receptors during *Salmonella* infection to determine whether endogenous immune responses contribute to bacterial clearance independently of, or synergistically with, bacteriophage therapy. Furthermore, where biological material remains available, stored RNA samples generated during this study could be analysed retrospectively to characterise immune activation following bacterial challenge and determine whether bacteriophage exposure itself elicits measurable changes in innate immune gene expression. Such analyses could be complemented by spatial localisation approaches, including in situ hybridisation and RNAscope, allowing immune gene expression to be visualised within specific tissues such as the gut epithelium, fat body and haemocytes. Integrating bacterial burden, phage abundance and host immune responses within the same experimental framework would provide a more complete understanding of the tripartite interactions between *Hermetia illucens*, *Salmonella* and bacteriophage, while informing the future optimisation of phage-based interventions for industrial BSFL production systems.

Collectively, this chapter establishes the first reproducible *Salmonella* Typhimurium infection model in *Hermetia illucens* suitable for evaluating bacteriophage intervention strategies. Although direct evidence of sustained in vivo phage replication was not obtained, prophylactic SPFM14 administration consistently reduced bacterial colonisation under several experimental conditions while maintaining high larval survival. These findings provide an important proof-of-concept for phage-based pathogen control in BSFL and establish an experimental platform for future studies integrating bacteriophage biology, host immunity and microbial ecology. Together with the experimental resources and datasets generated throughout this work, the findings presented here provide a strong foundation for optimising bacteriophage delivery strategies and improving microbiological safety within industrial BSFL production systems.

5.28. General Discussion and Conclusions

This thesis has established a foundation for understanding host-immunity and pathogen-bacteriophage interactions in *Hermetia illucens*. As the black soldier fly sector scales, there is a parallel need to integrate mechanistic biology with production-focused research. Alongside substantial advances in immunity, transcriptomics/genomics, and microbiome studies, this thesis contributes by (i) optimising BSFL experimental workflows, (ii) characterising long-term antimicrobial peptide expression following immune stimulation using qPCR, (iii) building a curated transcriptomic database to further characterise *H. illucens*, and (iv) linking phage-pathogen dynamics for the first time in the black soldier fly. Collectively, these strands of work underscore the value of combining technical optimisation with molecular and applied immunology. The following discussion examines how these findings intersect, how they align with current knowledge and how they may inform the future development of biologically informed, pathogen-resilient BSF production systems.

The experimental workflows developed in Chapter 2 allow for reproducible molecular methods and phage-pathogen study designs. Optimisation of larval handling, injection procedures, and RNA extraction significantly improved RNA yield and integrity, minimising mortality, degradation, and technical variability between replicates. These refinements ensured that extracted RNA was of sufficient quality for downstream applications including cDNA synthesis and qPCR analysis, with consistent amplification efficiency and clean melt curves. By reducing sample-to-sample noise at the earliest stages, these methodological improvements provided a reliable foundation for the immune mRNA abundance studies that followed. These protocols provide a foundation not only for this thesis but also for future phage and immune studies.

Our genome-guided, directional poly(A) transcriptome now extends beyond gene discovery to provide a systems-level framework for understanding innate immunity in *Hermetia illucens*. Integration of Gene Ontology, KEGG, Reactome and STRING protein-protein interaction analyses demonstrated that the identified immune genes form highly interconnected signalling networks centred on conserved Toll, IMD and JAK/STAT

pathways. Cross-pathway analyses further identified signalling hub genes including Relish, Kenny, Pelle, Hopscotch, Dorsal and PGRPs that repeatedly occurred across enriched biological pathways, highlighting their likely importance in coordinating immune responses. Rather than representing isolated immune components, these analyses demonstrate that BSFL immunity operates as an integrated signalling network comparable with other well-characterised dipteran systems.

BLASTP annotation demonstrated strong evolutionary conservation between *Hermetia illucens* and *Drosophila melanogaster*, supporting reliable functional inference of immune-associated genes despite the comparatively limited genomic resources currently available for BSFL. Functional enrichment consistently highlighted developmental, signalling and immune-associated biological processes, while cross-pathway analyses identified conserved signalling hubs centred on Toll, IMD and JAK/STAT pathway components. Although Reactome pathway enrichment did not survive false discovery rate correction, likely reflecting orthologue mapping limitations between *H. illucens* and model organisms, the convergence of multiple complementary analytical approaches provides strong evidence that the transcriptome captures the core molecular architecture of BSFL innate immunity.

Future work should focus on (i) tissue- and stage- resolved expression (fat body, gut, haemocytes to localise pathway use); (ii) RNAi/CRISPR to test necessity/sufficiency of key adaptors, kinases, and transcription factors; (iii) stimulus-specific challenges to map pathway selectivity; (iv) protein-level validation to confirm activation states; and (v) single-cell/spatial transcriptomics to resolve cell types and signalling niches.

This integrated immune-transcriptomic resource lays the groundwork for developing a BSFL immune landscape with the same mechanistic clarity that has been achieved in *Drosophila* (Ryckebusch, et al., 2025). Such advances will not only deepen our fundamental understanding of insect immunity but will also accelerate the development of biologically informed disease management strategies for large-scale insect farming.

Chapter 4 represents one of the first systematic efforts to characterise the late stages of antimicrobial peptide (AMP) expression in *H. illucens*, highlighting long-lasting response modules that protect larvae within microbial dense environments. By challenging larvae with defined PAMPs lipopolysaccharide and polyinosinic:polycytidylic acid (LPS; Poly(I:C)) and quantifying mRNA abundance, we showed sustained induction of multiple AMP families (defensin isoforms, cecropin-like, attacin paralogues) alongside PRR activation (TLR6). Overall, this supports a model in which BSFL deploy a broad, durable effector set, rather than a narrow approach. Dual activation of AMPs against bacterial and viral mimics points to convergent downstream activation (Toll/IMD cross-talk, JNK/JAK-STAT integration) that sustains transcriptional output up to 24 hours as seen here. Importantly, complementary multivariate analyses demonstrated that immune treatment, rather than biological replicate, was the principal driver of transcriptional variation. Heatmap visualisation, hierarchical clustering and principal component analysis identified distinct transcriptional response modules associated with antimicrobial peptide induction and pattern-recognition receptor activation, while regression and correlation analyses demonstrated that statistically significant dose-dependent responses were largely restricted to Defensin B-like and TLR6. Together, these analyses reveal that BSFL immunity is characterised by coordinated network-level transcriptional responses rather than independent regulation of individual genes.

One of the principal outputs of this thesis was the establishment of the first reproducible *Salmonella Typhimurium* infection model in *Hermetia illucens*. This model demonstrated sustained bacterial colonisation with minimal fitness costs to the larval host, providing an experimentally tractable platform for investigating pathogen persistence, innate immunity and bacteriophage intervention strategies. Using this model, prophylactic SPFM14 treatment reduced bacterial burden under several experimental conditions despite only transient phage persistence within the gut. By combining survival, CFU and PFU quantification, and time-course sampling, this work established a clear temporal profile of phage presence and bacterial reduction in larvae. The data show that phage titres peak within 2-8 hours post-administration and decline rapidly thereafter, with very low to undetectable levels beyond 24 hours. The rapid decline in plaque-forming units (PFU) is consistent with gut transit and

degradation rates in insect larvae, indicating that SPFM14 do not persist as a stable gut population under single-dose prophylaxis. However, persistence and impact may change with continuous or repeated input, protective formulations, or feed-based delivery that slows inactivation and improves mucosal contact. Together, these findings point to a complementary dynamic: phages act quickly to suppress initial bacterial burdens, potentially allowing the larval immune system to control or clear remaining bacteria once phage titres fall.

From an industrial perspective, these findings support the continued development of bacteriophage-based interventions as part of integrated pathogen management strategies for Black Soldier Fly production systems. Broadening therapeutic phage cocktails beyond *Salmonella*-targeting phages could further reduce bacterial contamination across larvae, feed substrates and frass while improving microbiological safety throughout production. Future studies should determine whether continuous or repeated phage delivery improves persistence within the larval gut, evaluate the stability of the gut microbiome following prolonged phage exposure, expand therapeutic phage panels against additional bacterial pathogens relevant to industrial BSFL production, and investigate formulation and feed-based delivery strategies capable of enhancing phage stability, persistence and efficacy. Together, these approaches have the potential to optimise phage-based biocontrol while supporting the sustainable expansion of the Black Soldier Fly industry.

Taken together, the findings from Chapters 2–5 demonstrate how methodological optimisation, systems-level transcriptomics, targeted immune profiling and bacteriophage intervention can be integrated to provide a mechanistic understanding of host–pathogen interactions in *Hermetia illucens*. Rather than representing independent studies, each chapter builds sequentially upon the previous one, culminating in an experimentally validated framework for investigating insect immunity and pathogen control within industrial BSFL production systems.

Beyond the individual findings presented throughout this work, this thesis has generated a series of experimental resources that provide a platform for future research

into *Hermetia illucens* biology, immunity and bacteriophage applications. These include a reproducible *Salmonella Typhimurium* infection model, a transcriptome-derived catalogue of immune-associated genes, validated qPCR assays targeting key innate immune effectors, comprehensive transcriptomic and qPCR datasets, optimised molecular workflows and associated biological material retained from immune stimulation and infection experiments where available. Collectively, these resources provide a foundation for future mechanistic and translational investigations that extend beyond the scope of the present thesis.

Key research questions arising from this thesis:

- How do antimicrobial peptide responses differ between individual BSFL tissues during bacterial infection?
- What are the temporal relationships between bacterial burden, phage abundance and innate immune activation during infection?
- Does bacteriophage exposure itself alter host innate immune signalling independently of bacterial killing?
- How do gut microbiome composition and diet influence immune priming and pathogen susceptibility?
- Can alternative phage formulations or repeated dosing improve persistence and efficacy within industrial BSFL production systems?
- Which transcriptomic hub genes identified in this thesis represent the most promising candidates for functional validation using RNAi, CRISPR or RNAscope?

Collectively, this thesis advances understanding of *Hermetia illucens* immunity, host-microbe interactions and bacteriophage biology while establishing experimental resources, molecular datasets and validated methodologies that will support future research and industrial application. By integrating transcriptomics, immune profiling and bacteriophage intervention within a single experimental framework, this work provides a comprehensive molecular investigation of BSFL immunity to date and

establishes a mechanistic foundation for the future development of biologically informed, pathogen-resilient Black Soldier Fly production systems.

Bibliography

- Abedon, S., Danis-Wlodarczyk, K., & Wozniak, D. (2021). Phage Cocktail Development for Bacteriophage Therapy: Toward Improving Spectrum of Activity Breadth and Depth. *Pharmaceuticals (Basel)*, 1019.
- Agaisse, H., Petersen, U. M., Boutros, M., Mathey-Prevot, B., & Perrimon, N. (2003). Signaling Role of Hemocytes in *Drosophila* JAK/STAT-Dependent Response to Septic Injury. *Developmental cell*, 441-450.
- Ahmed, S., Hussein, S., Qurbani, K., Ibrahim, R., Fareeq, A., Mahmood, K., & Mohamed, M. (2024). Antimicrobial resistance: Impacts, challenges, and future prospects. *Journal of Medicine, Surgery, and Public Health*, 100081.
- Albarella, D., Dall'Ara, P., Rossi, L., & Turin, L. (2025). Bacteriophage Therapy in Freshwater and Saltwater Aquaculture Species. *Bacteriophage Therapy in Freshwater and Saltwater Aquaculture Species*, 831.
- Aleksandr, M., George, C., Yan, R., Thomas, L. M., Richa, A., & Alejandro, A. S. (2008). Database Indexing for Production MegaBLAST Searches. *Bioinformatics*, 1757-1764.
- Alltech. (2022). *2022 Alltech Agri-Food Outlook reveals global feed production survey data and trends shaping the future*. Retrieved 07 2022, 26, from https://www.alltech.com/press-release/2022-alltech-agri-food-outlook-reveals-global-feed-production-survey-data-and-trends?utm_term=&utm_campaign=Ventisqueros+-+POP+-+2022&utm_source=adwords&utm_medium=ppc&hsa_acc=9535665100&hsa_cam=17623352286&hsa_grp=&h
- Anderson, K., Jurgens, G., & Nusslein-Volhard, C. (1985). Establishment of dorsal-ventral polarity in the *Drosophila* embryo: genetic studies on the role of the Toll gene product. *Cell*, 779–78.
- Antimicrobial Resistance Collaborators. (2021). Global burden of bacterial antimicrobial resistance 1990–2021: a systematic analysis with forecasts to 2050. *The Lancet*, 1199-1226.
- Anyega, A., Korir, N., Beesigamukama, D., Changeh, G., Nkoba, K., Subramanian, S., . . . Tanga, C. (2021). Black Soldier Fly-Composted Organic Fertilizer Enhances Growth, Yield, and Nutrient Quality of Three Key Vegetable Crops in Sub-Saharan Africa. *Front Plant Sci*.
- Applebaum, S., Ebstein, R., & Wyatt, G. (1966). Dissociation of ribosomal ribonucleic acid from silkworm pupae by heat and dimethylsulfoxide: Evidence for specific cleavage points. *Journal of Molecular Biology*, 29-41.
- Arbouzova, I. N., & Zeidler, P. M. (2006). JAK/STAT signalling in *Drosophila*: insights into conserved regulatory and cellular functions. *Development*, 2605-2616.
- Attigbo, F. A. (2019). Effectiveness of black soldier fly larvae in composting mercury contaminated organic waste. *Scientific African*, 6.
- Auger, L., Bouslama, S., Deschamps, M.-H. V., & Derome, N. (2023). Absence of microbiome triggers extensive changes in the transcriptional profile of *Hermetia illucens* during larval ontogeny. *Scientific Reports*, 2396.
- Banks, I., Gibson, W., & Cameron, M. (2014). Growth rates of black soldier fly larvae fed on fresh human faeces and their implication for improving sanitation. *Tropical medicine & international health*, 19(1), 14-22.
- Barragan-Fonseca, K. B., & Dicke, M. a. (2017). Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed – a review. *Journal of Insects as Food and Feed*, 105–120.

- Barragan-Fonseca, K., Dicke, M., & van Loon, J. (2017). Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed. *Journal of Insects as Food and Feed*, 3(2), 105-120.
- Barroso, F. G., de Haro, C., Sánchez-Muros, M. J., Venegas, E., Martínez-Sánchez, A., & Pérez-Bañón, C. (2019). The potential of various insect species for use as food for fish. *Aquaculture*, 514-522.
- Batinovic, S., Wassef, F., Knowler, S., Rice, D., Stanton, C., Rose, J., . . . Sobey, C. (2019). Bacteriophages in Natural and Artificial Environments. *Pathogens*, 100.
- Beesigamukama, D., Mochoge, B., Korir, N., Fiaboe, K., Nakimbugwe, D., Khamis, F., . . . Kelemu, S. (2020). Exploring black soldier fly frass as novel fertilizer for improved growth, yield, and nitrogen use efficiency of maize under field conditions. *Frontiers in Plant Science*, 11, 574592.
- Bekker, N., Heidelberg, S., Vestergaard, S., Nielsen, M., Riisgaard-Jensen, M., Zeuner, E., . . . Eriksen, N. (2021). Impact of substrate moisture content on growth and metabolic performance of black soldier fly larvae. *Waste Management*, 127, 73-79.
- Bell, J., Mullen, G., Leifer, C., Mazzoni, A., Davies, D., & Segal, D. (2003). Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends in immunology*, 528-533.
- Bella, J., Hindle, K., McEwan, P., & Lovell, S. (2008). The leucine-rich repeat structure. *Cellular and Molecular Life Sciences*, 2307-2333.
- Bertinetti, C., Samayoa, A., & Hwang, S. (2019). Effects of feeding adults of *Hermetia illucens* (Diptera: Stratiomyidae) on longevity, oviposition, and egg hatchability: insights into optimizing egg production. *Journal of insect science*, 1(19), 19.
- Better Origin. (2020). *WORLD'S FIRST FULLY AUTOMATED AI-POWERED INSECT FARM*. Retrieved 07 2022, 26, from <https://betterorigin.co.uk/2020/06/worlds-first-fully-automated-ai-powered-insect-mini-farm-is-set-to-revolutionise-the-food-industry/#:~:text=Better%20Origin%20has%20launched%20the,nutrients%20are%20fed%20to%20livestock>.
- Bharucha, J., Sun, L., Lu, W., Gartner, S., & Garzino-Demo, A. (2021). Human beta-defensin 2 and 3 inhibit HIV-1 replication in macrophages. *Frontiers in Cellular and Infection Microbiology*, 535352.
- Bian, G., Shin, S., Cheon, H., Kokoza, V., & Raikhel, A. (2005). Transgenic alteration of Toll immune pathway in the female mosquito *Aedes aegypti*. *PNAS*, 13568-13573.
- Bin Hafeez, A., Jiang, X., Bergen, P., & Zhu, Y. (2021). Antimicrobial Peptides: An Update on Classifications and Databases. *MDPI*, 11691.
- Bina, S., & Zeidler, M. (2013). JAK/STAT Pathway Signalling in *Drosophila Melanogaster*. *n Madame Curie Bioscience Database [Internet]*.
- Binari, R., & Perrimon, N. (1994). Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in *Drosophila*. *Genes & Development*, 300-312.
- Biswas, B., Adhya, S., Washart, P., Paul, B., Trostel, A., Powell, B., . . . Merrill, C. (2002). Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. *Infection and immunity*, 204-210.
- Blandin, S., Moita, L., Köcher, T., Wilm, M., Kafatos, F., & Levashina, E. (2002). Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the Defensin gene. *EMBO reports*.
- Bogner, J., Pipatti, R., Hashimoto, S., Diaz, C., Mareckova, K., Diaz, L., . . . and Zhang, T. (2008). Mitigation of global greenhouse gas emissions from waste: conclusions and strategies from the Intergovernmental Panel on Climate Change (IPCC) Fourth Assessment Report. Working Group III (Mitigation). *Waste Management and Research*, 26(1), 11-32.

- Bonelli, M., Bruno, D., Brilli, M., Gianfranceschi, N., Tian, L., Tettamanti, G., . . . Casartelli, M. (2020). Black Soldier Fly Larvae Adapt to Different Food Substrates through Morphological and Functional Responses of the Midgut. *Int J Mol Sci*, 4955.
- Bonelli, M., Bruno, D., Brilli, M., Gianfranceschi, N., Tian, L., Tettamanti, G., . . . and Casartelli, M. (2020). Black Soldier Fly Larvae Adapt to Different Food Substrates through Morphological and Functional Responses of the Midgut. *International Journal of Molecular Sciences*, 21(14), 4955.
- Bonelli, M., Bruno, D., Brilli, M., Gianfranceschi, N., Tian, L., Tettamanti, G., . . . Casartelli, M. (2020). Black Soldier Fly Larvae Adapt to Different Food Substrates through Morphological and Functional Responses of the Midgut. *Int J Mol Sci*.
- Bonelli, M., Bruno, D., Caccia, S., Sgambetterra, G., Cappellosa, S., Jucker, C., . . . Casartelli, M. (2019). Structural and Functional Characterization of *Hermetia illucens* Larval Midgut. *Sec. Invertebrate Physiology*.
- Booth, D., & Sheppard, C. (1984). Oviposition of the Black Soldier Fly, *Hermetia illucens* (Diptera: Stratiomyidae): Eggs, Masses, Timing, and Site Characteristics. *Environmental Entomology*, 13(2), 421-423.
- Brady, D., Grapputo, A., Romoli, O., & Sandrelli, F. (2019). Insect cecropins, antimicrobial peptides with potential therapeutic applications. *International journal of molecular sciences*, 5862.
- Briscoe, A., & Chittka, L. (2001). The evolution of color vision in insects. *Annual Review of Entomology*, 49, 471-510 .
- Brown, S., Hu, N., & Hombria, J. G. (2001). Identification of the first invertebrate interleukin JAK/STAT receptor, the *Drosophila* gene *domeless*. *Current Biology*, 1700-1705.
- Bruni, L., Pastorelli, R., Viti, C., Gasco, L., & Parisi, G. (2018). Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with *Hermetia illucens* (black soldier fly) partially defatted larvae meal as partial dietary protein source. *Aquaculture*, 56-63.
- Bruno, D., Bonelli, M., De Filippis, F., Di Lelio, I., Tettamanti, G., Casartelli, M., . . . Caccia, S. (2019). The Intestinal Microbiota of *Hermetia illucens* Larvae Is Affected by Diet and Shows a Diverse Composition in the Different Midgut Regions. *Appl Environ Microbiol.*, e01864-18.
- Bruno, D., Mohamed, A., Al-Akeel, R., Alharbi, H., Tettamanti, G., & Eleftherianos, I. (2026). Immunity of the black soldier fly (*Hermetia illucens*): recent progress and technological applications. *J Invertebr Pathol*.
- Bruno, D., Montali, A., Mastore, M., Brivio, M., Mohamed, A., Tian, L., . . . Tettamanti, G. (2021). nsights Into the Immune Response of the Black Soldier Fly Larvae to Bacteria. . *Front. Immunol*.
- Buchon, N., Broderick, A. N., Poidevin, M. P., & Lemaitre, B. (2009). *Drosophila* intestinal response to bacterial infection: activation of host defense and stem cell proliferation. *Cell Host Microbe*, 200-211.
- Buchon, N., Silverman, N., & Cherry, S. (2014). Immunity in *Drosophila melanogaster* — from microbial recognition to whole-organism physiology. *Nature reviews immunology*, 796-810.
- Buchon, N., Silverman, N., & Cherry, S. (2014). Immunity in *Drosophila melanogaster*--from microbial recognition to whole-organism physiology. *Nat Rev Immunol*, 796-810.
- Bukchin-Peles, S., Lozneva, K., Tomberlin, J., & Zilberman, D. (2025). From waste management to protein innovation: Black soldier fly as an embodiment of the circular bioeconomy. *Future Foods* , 100592.
- Bulet, P., Hetru, C., Dimarcq, J. L., & Hoffmann, D. (1999). Antimicrobial peptides in insects; structure and function. *Developmental & Comparative Immunology*, 329-344.

- Bulet, P., Stöcklin, R., & Menin, L. (2004). Anti-microbial peptides: from invertebrates to vertebrates. *Immunol Rev*, 169-184.
- Bulet, P., Stöcklin, R., & Menin, L. (2004). Anti-microbial peptides: from invertebrates to vertebrates. *Immunol Rev*.
- Burkhard, P., Stetefeld, J., & Strelkov, S. (2001). Coiled coils: a highly versatile protein folding motif. *Trends in cell biology*, 82-88.
- Bustin, S., Beaulieu, J., Huggett, J., Jaggi, R., Kibenge, F., Olsvik, P., . . . Toegel, S. (2010). MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC molecular biology*, 74.
- Cai, M., Ma, S., Hu, R., Tomberlin, J., Yu, C., Huang, Y., . . . and Zhang, J. (2018). Systematic characterization and proposed pathway of tetracycline degradation in solid waste treatment by *Hermetia illucens* with intestinal microbiota. *Environmental Pollution*, 242, 634-642.
- Caimi, C., Renna, M., Sogari, G., & Gasco, L. (2021). Insect-based feed ingredients for aquafeeds: A global scenario. *Aquaculture Reports*, 100805.
- Candian, V., Meneguz, M., & Tedeschi, R. (2023). *Immune Responses of the Black Soldier Fly Hermetia illucens (L.) (Diptera: Stratiomyidae) Reared on Catering Waste*. Italy: Life.
- Capparelli, R., Parlato, M., Borriello, G., Salvatore, P., & Iannelli, D. (2007). Experimental phage therapy against *Staphylococcus aureus* in mice. *Antimicrobial agents and chemotherapy*, 2765-2773.
- Carlsson, A., Engström, P., Palva, E., & Bennich, H. (1991). Attacin, an antibacterial protein from *Hyalophora cecropia*, inhibits synthesis of outer membrane proteins in *Escherichia coli* by interfering with *omp* gene transcription. *Infection and Immunity*, 3040-3045.
- CDC. (2024). How Salmonella Infections Happen. *CDC*.
- Cerenius, L., & Söderhäll, K. (2004). The prophenoloxidase-activating system in invertebrates. *Immunol Rev*.
- CFIA. (2022). *Insects as Livestock Feed*. Retrieved from Canadian Food Inspection Agency: <https://inspection.canada.ca>
- Chapman, R. (1998). The insects: structure and function. *Cambridge university press*.
- Cheng, J., Chiu, S., & Lo, I. (2017). Effects of moisture content of food waste on residue separation, larval growth and larval survival in black soldier fly bioconversion. *Waste Management*, 67, 315-323.
- Chia, S., Tanga, C., Khamis, F., Mohamed, S., Salifu, D., Sevgan, S., . . . Ekesi, S. (2018). Threshold temperatures and thermal requirements of black soldier fly *Hermetia illucens*: Implications for mass production. *PLoS One*, 13(11).
- Chia, S., Tanga, C., Khamis, F., Mohamed, S., Salifu, D., Sevgan, S., . . . Ekesi, S. (2018). Threshold temperatures and thermal requirements of black soldier fly *Hermetia illucens*: Implications for mass production. *PLoS One*, e0206097.
- Chia, Y. S., Tanga, M. C., Khamis, M. F., Mohamed, H. S., Salifu, D., Sevgan, S., . . . Ekesi, S. (2018). Threshold temperatures and thermal requirements of black soldier fly *Hermetia illucens*: Implications for mass production. *PLoS One*.
- Cho, S., Kim, C., & Kim, M. (2020). Effects of microplastics and salinity on food waste processing by black soldier fly (*Hermetia illucens*) larvae. *Journal of Ecology and Environment*, 44.
- Choi, S. C., Silva, T. M., Song, C. H., & Lee, S. D. (2013). Antimicrobial peptides as a promising alternative to conventional antibiotics in livestock and poultry.

- Antimicrobial peptides as a promising alternative to conventional antibiotics in livestock and poultry*, 561-572.
- Chu, H., Pazgier, M., Jung, G., Nuccio, S., Castillo, P., De Jong, M., . . . Salzman, N. (2012). Human α -defensin 6 promotes mucosal innate immunity through self-assembled peptide nanonets. *Science*, 477-481.
- Chuang, Y. C., Chen, J. Y., Lin, T. L., & Chang, Y. H. (2011). Enhancing resistance to *Vibrio vulnificus* infection and modulation of innate immunity by antimicrobial peptide epinecidin-1 in mice. *Antimicrobial Agents and Chemotherapy*, 5881-5896.
- Cifuentes, Y., Vilcinskis, A., Kämpfer, P., & Glaeser, S. (2022). Isolation of *Hermetia illucens* larvae core gut microbiota by two different cultivation strategies. *Antonie Van Leeuwenhoek*, 821-837.
- Clayton, M. A., Dong, Y., & Dimopoulos, G. (2013). The Anopheles Innate Immune System in the Defense against Malaria Infection. *J Innate Immun.*, 169-181.
- Clokier, M., Millard, A., Letarov, A., & Heaphy, S. (2011). Phages in nature. *Bacteriophage*, 31-45.
- Cornet, B., Bonmatin, J., Hetru, C., Hoffmann, J., Ptak, M., & Vovelle, F. (1995). Refined three-dimensional solution structure of insect defensin A. *Structure*, 435-448.
- Cornet, B., Bonmatin, J., Hetru, C., Hoffmann, J., Ptak, M., & Vovelle, F. (1995). Refined three-dimensional solution structure of insect defensin A. *Structure*, 435-448.
- Costa, F., Gomes, D., Reis, R. L., & Martins, M. C. (2019). Antimicrobial peptides-based materials: a step forward to prevent biofilm-related infections. *Biotechnology Advances*, 107374.
- Costagli, S., Abenaim, L., Rosini, G., Conti, B., & Giovannoni, R. (2024). De Novo Genome Assembly at chromosome-scale of *Hermetia illucens* (Diptera Stratiomyidae) via PacBio and Omni-C proximity ligation technology. *Insects*, 133.
- Cresti, L., Cappello, G., & Pini, A. (2024). Antimicrobial peptides towards clinical application—A long history to be concluded. *International Journal of Molecular Sciences*, 4870.
- Crow, K., & Wagner, G. (2006). What Is the Role of Genome Duplication in the Evolution of Complexity and Diversity? *Molecular Biology and Evolution*, 887–892.
- Czajkowski, R., Roca, A., & Matilla, M. (2025). Harnessing Bacteriophages for Sustainable Crop Protection in the Face of Climate Change. *Microb Biotechnol*, e70108.
- Da Silva, G., & Hesselberg, T. (2019). ; A Review of the Use of Black Soldier Fly Larvae, *Hermetia illucens* (Diptera: Stratiomyidae), to Compost Organic Waste in Tropical Regions. *Neotropical Entomology* , 49, 151-162.
- De Smet, J., Vandeweyer, D., Van Moll, L., Lachi, D., & Van Campenhout, L. (2021). Dynamics of *Salmonella* inoculated during rearing of black soldier fly larvae (*Hermetia illucens*). *Dynamics of Salmonella inoculated during rearing of black soldier fly larvae (Hermetia illucens)*, 110692.
- de Souza-Vilela, J., Andrew, N., & Ruhnke, I. (2019). Insect protein in animal nutrition. *Animal Production Science*, 51(11), 2029-2036.
- Dedrick, R., Guerrero-Bustamante, C., Garlena, R., Russell, D., Ford, K., Harris, K., . . . Hatfull, G. (2019). Engineered bacteriophages for treatment of a patient with a disseminated drug resistant *Mycobacterium abscessus*. *J. Gen. Microbiol.*, 730-733.
- DeLeo, D., Pérez-Moreno, J., Vázquez-Miranda, H., & Bracken-Grissom, H. (2018). RNA profile diversity across arthropoda: guidelines, methodological artifacts, and expected outcomes. *Biology Methods and Protocols*, 012.
- DeRaedt, J. M. (2022). microRNA profile of *Hermetia illucens* (black soldier fly) and its implications on mass rearing. *PLOS ONE*.

- Dho, M., Candian, V., & Tedeschi, R. (2023). Insect antimicrobial peptides: advancements, enhancements and new challenges. *Antibiotics*, 952.
- Diener, S., Studt Solano, N., Roa Gutiérrez, F., Zurbrügg, C., & Tockner, K. (2011). Biological treatment of municipal organic waste using black soldier fly larvae. *Waste and Biomass Valorization volume*, 2, 357–363.
- Diener, S., Zurbrügg, C., & Tockner, K. (2009). Conversion of organic material by black soldier fly larvae: Establishing optimal feeding rates. . *Waste Manag. Res.*, 27, 603-610.
- Diener, S., Zurbrügg, C., & Tockner, K. (2015). Bioaccumulation of heavy metals in the black soldier fly, *Hermetia illucens* and effects on its life cycle. *Journal of Insects as Food and Feed*(4), 261-270.
- Diener, S., Zurbrügg, C., Roa Gutiérrez, F., Hong, N. D., Morel, A., Koottatep, T., & Tockner, K. (2011). Black soldier fly larvae for organic waste treatment – prospects and constraints. *2nd International Conference on Solid Waste Management in Developing Countries*, 52-59.
- d’Herelle, F. (1917). Sur un microbe invisible antagoniste des bacilles.
- Dimarcq, J., Zachary, D., Hoffmann, J., Hoffmann, D., & Reichhart, J. (1990). Insect immunity: expression of the two major inducible antibacterial peptides, defensin and dipterecin, in *Phormia terranova*. *The EMBO Journal*, 2507-2515.
- Dolan, J., Walshe, K., Alsbury, S., Hokamp, K., O’Keeffe, S., Okafuji, T., . . . Mitchell, K. (2007). The extracellular leucine-rich repeat superfamily; a comparative survey and analysis of evolutionary relationships and expression patterns. *BMC genomics*, 320.
- Dong, Y., Das, S., Cirimotich, S.-N. A., McLean, K., & Dimopoulos, G. (2011). Engineered *Anopheles* Immunity to *Plasmodium* Infection. *PLoS*.
- Duan, T., Du, Y., Xing, C., Wang, H., & Wang, R. (2022). Toll-like receptor signaling and its role in cell-mediated immunity. *Frontiers in immunology*, 812774.
- Duckert, P., Brunak, S., & Blom, N. (2004). Prediction of proprotein convertase cleavage sites. *Protein Engineering Design and Selection*, 107-112.
- Dyson, H., & Wright, P. (2005). Intrinsically unstructured proteins and their functions. *Intrinsically unstructured proteins and their functions.*, 197-208.
- EFSA and ECDC. (2024). The European union one health 2023 zoonoses report. *EFSA*, 9106.
- EFSA Panel on Nutrition, Novel Foods and Food Allergens. (2021). Scientific opinion on the safety of frozen and dried formulations from whole house crickets (*Acheta domesticus*) as a novel food. *EFSA Journal*, e06317.
- Ekas, L. A., Cardozo, T. J., Flaherty, M. S., McMillan, E. A., Gonsalves, F. C., & Bach, E. A. (2010). Characterization of a dominant-active STAT that promotes tumorigenesis in *Drosophila*. *Developmental Biology*, 621-636.
- Eke, M., Tougeron, K., Hamidovic, A., L, T. S., Hance, T., & Renoz, F. (2023). Deciphering the functional diversity of the gut microbiota of the black soldier fly (*Hermetia illucens*): recent advances and future challenges. *Anim Microbiome*.
- Elhag, O., Zhang, Y., Xiao, X., Cai, M., Zheng, L., Jordan, H., . . . Zhang, J. (2022). Inhibition of Zoonotic Pathogens Naturally Found in Pig Manure by Black Soldier Fly Larvae and Their Intestine Bacteria. *Insects*.
- Elhag, O., Zhou, D., Song, Q., Soomro, A., Cai, M., Zheng, L., . . . Zhang, J. (2017). Screening, expression, purification and functional characterization of novel antimicrobial peptide genes from *Hermetia illucens* (L.). *PloS one*, 0169582.
- Elissen, H. (2007). Sludge reduction by aquatic worms in wastewater treatment: with emphasis on the potential application of *Lumbriculus variegatus*.

- Elshamy, A., Kamal, S., Mahmoud, M., Elhasany, A., Shady, A., Mohamed, S., . . . El-Hady, S. (2025). Recent insights on phage therapy against multidrug-resistant *Acinetobacter baumannii*. *Recent insights on phage therapy against multidrug-resistant Acinetobacter baumannii*, 1-16.
- El-Zayat, S., Sibaii, H., & Mannaa, F. (2019). Toll-like receptors activation, signaling, and targeting: an overview. *Bulletin of the National Research Centre*, 1-12.
- Erickson, M., Islam, M., Sheppard, C., Liao, J., & Doyle, M. (2004). *Escherichia coli* O157: H7 and *Salmonella enterica* serovar enteritidis in chicken manure by larvae of the black soldier fly. *J Food Prot*, 67(4), 685-90.
- European Commission. (2009). *Regulation (EC) No 1069/2009 of the European Parliament and of the Council*. Brussels: Official Journal of the European Union.
- Fahmy, L. (2023). *Identification and recombinant production of antimicrobial peptides from the Black Soldier Fly, Hermetia illucens (L.)*. Cambridge: Doctoral Dissertation.
- Fahmy, L., Ali, Y., Seilly, D., McCoy, R., Owens, R., Pipan, M., . . . Grant, A. (2023). An attacin antimicrobial peptide, Hill_BB_C10074, from *Hermetia illucens* with anti-*Pseudomonas aeruginosa* activity. *BMC microbiology*, 378.
- Fahmy, L., Generalovic, T., Ali, Y., Seilly, D., Sivanesan, K., Kalmar, L., . . . Grant, A. (2024). A novel family of defensin-like peptides from *Hermetia illucens* with antibacterial properties. *BMC microbiology*, 167.
- FAO. (2009). FAOs director-general on how to feed the world in 2050. *Popul Dev Rev.*, 35(4), 837-839.
- FAO. (2021). Looking at edible insects for food and feed security. (*Food and Agriculture Organization of the United Nations*).
- FAO. (2022). *Livestock and Landscapes*. Retrieved 07 26, 2022, from <https://www.fao.org/3/ar591e/ar591e.pdf>
- FAO. (2022(b)). *The State of World Fisheries and Aquaculture*. FAO.
- Farhana, A., & Khan, Y. (2023). Biochemistry, Lipopolysaccharide. *StatPearls Publishing*.
- Farming Connect. (2022). *Potential sources of protein for animal feed: Insects*. Retrieved 07 2022, 26, from <https://businesswales.gov.wales/farmingconnect/news-and-events/technical-articles/potential-sources-protein-animal-feed-insects>
- Fawole, F. J. (2021(b)). Evaluation of defatted black soldier fly larvae meal and oil as sustainable feed ingredients in aquaculture diets. *Aquaculture Nutrition*, 786–800.
- Fawole, J. (2022). Nutritional evaluation of black soldier fly larvae meal as a replacement for fishmeal in aquafeeds: A review. *Aquaculture Reports*, 00957.
- Febvre, H., Rao, S., Gindin, M., Goodwin, N., Finer, E., Vivanco, J., . . . Weir, T. (2019). PHAGE study: effects of supplemental bacteriophage intake on inflammation and gut microbiota in healthy adults. *Nutrients*, 666.
- Feng, M., Fei, S., Xia, J., Labropoulou, V., Swevers, L., & Sun, J. (2020). Antimicrobial Peptides as Potential Antiviral Factors in Insect Antiviral Immune Response. *Frontiers in immunology*, 2030.
- Feng, X., Memon, F., Ma, C., Zhu, J., Huang, Z., Tettamanti, G., . . . Tian, L. (2024). Proteomic and transcriptomic analysis of cold- and heat-tolerant black soldier fly (*Hermetia illucens*) larvae. *Journal of Insect Physiology*.
- Ferry, T., Le Bouar, M., Briot, T., Roussel-Gaillard, T., Perpoint, T., Roux, S., . . . Ndiaye, M. (2024). Access to phage therapy at Hospices Civils de Lyon in 2022: Implementation of the PHAGEinLYON Clinic programme. *International Journal of Antimicrobial Agents*, 107372.
- Figueras, A. (2024). A global call to action on antimicrobial resistance at the UN General Assembly. *Antibiotics*, 915.

- Fitriana, E., Laconi, E., Astuti, D., & Jayanegara, A. (2022). Effects of various organic substrates on growth performance and nutrient composition of black soldier fly larvae: A meta-analysis. *Bioresource Technology Reports*, 18.
- Fjell, C. D., Hiss, J. A., Hancock, R. E., & Schneider, G. (2012). Designing antimicrobial peptides: form follows function. *Nature Reviews Drug Discovery*, 37-51.
- Fleige, S., & Pfaffl, M. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular aspects of medicine*, 126-139.
- Foley, J., Steinmetz, G., & Richardson, J. (2022). *The future of Food*. Retrieved 07 28, 2022, from <https://www.nationalgeographic.com/foodfeatures/feeding-9-billion/>
- Frolet, C., Thoma, M., Blandin, S., Hoffmann, J., & Levashina, E. (2006). Boosting NF-kappaB-dependent basal immunity of *Anopheles gambiae* aborts development of *Plasmodium berghei*. *Immunity*, 677-85.
- FSA. (2023). *Food Standards Agency*. Retrieved from Insects in feed: UK position post-Brexit: <https://www.food.gov.uk>
- Fu, J., Zong, X., Jin, M., Min, J., Wang, F., & Wang, Y. (2023). Mechanisms and regulation of defensins in host defense. *Signal Transduction and Targeted Therapy*, 300.
- Fujiwara, H., & Ishikawa, H. (1986). Molecular mechanism of introduction of the hidden break into the 28S rRNA of insects: implication based on structural studies. *Nucleic acids research*, 6393-6401.
- Funderburg, N., Lederman, M., Feng, Z., Drage, M., Jadowsky, J., Harding, C., . . . Sieg, S. (2007). Human β -defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. *Proceedings of the National Academy of Sciences*, 18631-18635.
- Furman, D., Young, R., & Catts, P. (1959). *Hermetia illucens* (Linnaeus) as a factor in the natural control of *Musca domestica* Linnaeus. *Journal of Economic Entomology*, 52(5), 917-921.
- Galassi, G., Jucker, C., Parma, P., Lupi, D., Crovetto, G., Savoldelli, S., & Colombini, S. (2021). Impact of Agro-industrial Byproducts on Bioconversion, Chemical Composition, in vitro Digestibility, and Microbiota of the Black Soldier Fly (Diptera: Stratiomyidae) Larvae. *Journal of Insect Science*, 21(1), 8.
- Ganz, T. S., Daher, K., Bainton, D., & Lehrer, R. (1985). Defensins. Natural peptide antibiotics of human neutrophils. *The Journal of clinical investigation*, 1427-1435.
- Gasco, L., Biancarosa, I., & Liland, N. (2020). From waste to feed: A review of recent knowledge on insects as producers of protein and fat for animal feeds. *Current Opinion in Green and Sustainable Chemistry*, 67-79.
- Gasco, L., Finke, M., & Van Huis, A. (2018). Can diets containing insects promote animal health? *Journal of Insects as Food and Feed*, 4(1), 1-4.
- Gasco, L., Finke, M., & Van Huis, A. (2020). Insects as Animal Feed: Novel Ingredients for Use in Pet, Aquaculture and Livestock Diets. *Springer*.
- Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R., & Bairoch, A. (2003). ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res*, 3784-3788.
- Generalovic, T. N., McCarthy, S. A., Warren, I. A., Wood, J. M., Torrance, J., Sims, Y., . . . & Jiggins, C. D. (2021). A high-quality, chromosome-level genome assembly of the Black Soldier Fly (*Hermetia illucens* L.). *G3: Genes, Genomes, Genetics*.
- Generalovic, T., McCarthy, A. S., Warren, A. I., Wood, M. J., Torrance, J., Sims, Y., . . . Jiggins, D. C. (2021). *A high-quality, chromosome-level genome assembly of the Black Soldier Fly (Hermetia illucens L.)*. Oxford University Press.
- George, C. (1998). Hirudin, heparin and Heinrich Necheles. *Nephrology*, 225-228.

- Giannetti, D., Schifani, E., Reggiani, R., Mazzoni, E., Reguzzi, M., Castracani, C., . . . Grasso, D. (2022). Do It by Yourself: Larval Locomotion in the Black Soldier Fly *Hermetia illucens*, with a Novel “Self-Harvesting” Method to Separate Prepupae. *Insects*, 127.
- Girma, A. (2025). Bacteriophages as an alternative strategy for the treatment of drug resistant bacterial infections: Current approaches and future perspectives. *Cell Surf*, 100149.
- Giunti, G., Campolo, O., Laudani, F., & Palmeri, V. (2018). Male courtship behaviour and potential for female mate choice in the black soldier fly *Hermetia illucens* L.(Diptera: Stratiomyidae). *Entomologia Generalis*, 38(1), 29-46.
- Gligorescu, A., Toepfl, S., Heinz, V., & Czerny, M. (2022). Black soldier fly as a future food: An overview of processing, safety, and regulation. *Frontiers in Sustainable Food Systems*, 875498.
- Global price of Fish Meal*. (2025, March 11). Retrieved from Fred: https://fred.stlouisfed.org/series/PFISHUSDM?utm_source=chatgpt.com
- GOV.UK. (2025). Supply unlicensed medicinal products (specials). *Medicines and Healthcare products Regulatory Agency*.
- Green, T., & Popa, R. (2012). Enhanced Ammonia Content in Compost Leachate Processed by Black Soldier Fly Larvae. *Applied Biochemistry and Biotechnology*, 166(6), 1381-7.
- Greer, S., Rabiey, M., Studholme, D., & Grant, M. (2025). The potential of bacteriocins and bacteriophages to control bacterial disease of crops with a focus on *Xanthomonas* spp. *Journal of the Royal Society of New Zealand*, 302-326.
- Hagens, S., Habel, A., Von Ahsen, U., Von Gabain, A., & Bläsi, U. (2004). Therapy of experimental *Pseudomonas* infections with a nonreplicating genetically modified phage. *Antimicrobial agents and chemotherapy*, 3817-3822.
- Halloran, A., Roos, N., Eilenberg, J., Cerutti, A., & Bruun, S. (2018). Life cycle assessment of edible insects for food and feed in Thailand. *Journal of Cleaner Production*, 1100-1110.
- Harrison, D. M., Binari, R., Gilman, M., & Perrimon, N. (1998). *Drosophila* unpaired encodes a secreted protein that activates the JAK signaling pathway. *Genes & Development*, 3252-3263.
- Hashimoto, C., Hudson, K., & Anderson, K. (1988). The Toll gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell*, 269-279.
- Haycraft, B. J. (1883). IV. On the action of a secretion obtained from the medicinal leech on the coagulation of the blood. *Royal Society Publishing*.
- He, L., Zhang, C., Yang, H., Ding, B., Yang, H., & Zhang, S. (2023). Characterization and functional analysis of toll receptor genes during antibacterial immunity in the green peach aphid *Myzus persicae* (Sulzer). *Insects*, 275.
- He, Y., Jia, Q., Cai, K., Xu, S., Li, H., Xie, Q., . . . Jiao, X. (2025). The global, regional, and national burden of Invasive Non-typhoidal *Salmonella* (iNTS): An analysis from the Global Burden of Disease Study 1990–2021. *PLOS*, e0012960.
- Head, R. S., Komori, K., LaMere, A. S., Whisenant, T., Van Nieuwerburgh, F., Salomon, R. D., & Ordoukhanian, P. (2014). Library construction for next-generation sequencing: Overviews and challenges. *BioTechniques*, 61-77.
- Hegedus, D., Erlandson, M., Gillott, C., & Toprak, U. (2009). New insights into peritrophic matrix synthesis, architecture, and function. *Annu Rev Entomol.*, 285-302.
- Henry, M., Gasco, L., Piccolo, G., & Fountoulaki, E. (2022). Review on the use of insects in the diet of farmed fish: Past and future. *Animal Feed Science and Technology*.

- Henry, M., Gasco, L., Piccolo, G., & Fountoulaki, E. (2023). Insect meals in aquafeeds: A review of the current state of knowledge. *Trends in Food Science & Technology*, 252–264.
- Heussler, C., Walter, A., Oberkofler, H., Insam, H., Arthofer, W., Schlick-Steiner, B., & Steiner, F. (2018). Influence of three artificial light sources on oviposition and half-life of the Black Soldier Fly, *Hermetia illucens* (Diptera: Stratiomyidae): Improving small-scale indoor rearing. *PLoS One*, 13(5).
- Hoffmann, J. A., & Reichhart, J. M. (2002). *Drosophila* innate immunity: an evolutionary perspective. *Nature Immunology*, 121-126.
- Holmes, L., Vanlaerhoven, S., & Tomberlin, J. (2012). Relative humidity effects on the life history of *Hermetia illucens* (Diptera: Stratiomyidae). *Environmental entomology*, 41(4), 971-978.
- Hong, T., & Medzhitov, R. (2001). *Drosophila* MyD88 is an adapter in the Toll signaling pathway. *Proceedings of the National Academy of Sciences*, 12654-12658.
- Hou, X. S., Melnick, M. B., & Perrimon, N. (1996). Marelle acts downstream of the *Drosophila* HOP/JAK kinase and encodes a protein similar to the mammalian STATs. *Cell*, 411-419.
- Hu, C., Yang, L., Wang, H., Xiao, X., Wang, Z., Gong, X., . . . Li, W. (2023). Analysis of heavy metals in the conversion of lake sediment and restaurant waste by black soldier fly (*Hermetia illucens*). *Front Bioeng Biotechnol*.
- Hull, J., Alexander, M., Bruneaux, M., & Christoffels, A. (2022). Gene expression differentials driven by mass rearing and artificial selection in black soldier fly (*Hermetia illucens*). *Insect Molecular Biology*, 756-776.
- Hultmark, D., Engström, A., Andersson, K., Steiner, H., Bennich, H., & Boman, H. (1983). Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia*. *The EMBO journal*, 571-576.
- Hwang, J., Lee, J., Kim, Y., Bang, H., Yun, E., Kim, S., . . . Kim, I. (2009). Isolation and characterization of a defensin-like peptide (Coprinsin) from the dung beetle, *Copris tripartitus*. *International journal of peptides*, 136284.
- Ignasiak, K., & Maxwell, A. (2017). *Galleria mellonella* (greater wax moth) larvae as a model for antibiotic susceptibility testing and acute toxicity trials. *BMC research*, 428.
- Ijdema, F., De Smet, J., Crauwels, S., Lievens, B., & Van Campenhout, L. (2022). Meta-analysis of larvae of the black soldier fly (*Hermetia illucens*) microbiota based on 16S rRNA gene amplicon sequencing. *FEMS Microbiology Ecology*, fiac094.
- Ishikawa, H., & Newburgh, R. (1972). Studies of the thermal conversion of 28 S RNA of *Galleria mellonella* (L.) to an 18 S product. *Journal of Molecular Biology*, 135-144.
- Iswa, U., & Wilson, D. (2015). *Global waste management outlook. International Solid Waste Association (ISWA) and United Nations Environmental Programme (UNEP), Nairobi.* UN environment programme.
- Jacoby, C. (1904). Hirudin. *Deutsche Medizinische Wochenschrift*.
- Jang, H., Bae, H., & Cho, Y. (2019). Exploitation of *Drosophila* Infection Models to Evaluate Antibacterial Efficacy of Phages. *Methods Mol Biol*, 183-190.
- Jens, R., & Reynolds, S. (2009). Insect Infection and Immunity: Evolution, Ecology, and Mechanisms. *Oxford Academic*.
- Jeon, J., & Yong, D. (2019). Two Novel Bacteriophages Improve Survival in *Galleria mellonella* Infection and Mouse Acute Pneumonia Models Infected with Extensively Drug-Resistant *Pseudomonas aeruginosa*. *Appl Environ Microbiol*, e02900-18.
- Jowett, T. (1986). Preparation of nucleic acids. In: Roberts, D.B. (ed.) *Drosophila: A Practical Approach*. . Oxford: IRL Press, 275-286.

- Kaczoe, M., Bulak, P., Proc-Pietrycha, K., Kirichenko-Babko, M., & Bieganski, A. (2022). The Variety of Applications of *Hermetia illucens* in Industrial and Agricultural Areas—Review. *Biology (Basel)*.
- Kalsy, M., Tonk, M., Hardt, M., Dobrindt, U., Zdybicka-Barabas, A., Cytrynska, M., . . . Mukherjee, K. (2020). The insect antimicrobial peptide cecropin A disrupts uropathogenic *Escherichia coli* biofilms. *npj Biofilms and Microbiomes*, 6.
- Kardas, P., Devine, S., Golembesky, A., & Roberts, C. (2005). A systematic review and meta-analysis of misuse of antibiotic therapies in the community. *International journal of antimicrobial agents*, 106-113.
- Kariuki, E., Kibet, C., Paredes, J., Mboowa, G., Mwaura, O., Njogu, J., . . . Tanga, C. (2023). Metatranscriptomic analysis of the gut microbiome of black soldier fly larvae reared on lignocellulose-rich fiber diets unveils key lignocellulolytic enzymes. *Front. Microbiol.*
- Katsuyama, T., Comoglio, F., Seimiya, M., Cabuy, E., & Paro, R. (2015). During *Drosophila* disc regeneration, JAK/STAT coordinates cell proliferation with Dilp8-mediated developmental delay. *Proceedings of the National Academy of Sciences*, 2327-E2336.
- Kazemzadeh-Narbat, M., Cheng, H., Chabok, R., Alvarez, M., De La Fuente-Nunez, C., Phillips, K., & Khademhosseini, A. (2021). Strategies for antimicrobial peptide coatings on medical devices: A review and regulatory science perspective. *Critical reviews in biotechnology*, 94-120.
- Kelly, L., & Jameson, E. (2024). Bacteriophage cocktail shows no toxicity and improves the survival of *Galleria mellonella* infected with *Klebsiella* spp. *Journal of Virology*, e00272-24.
- Khan, M., & Rahman, S. (2022). Use of Phages to Treat Antimicrobial-Resistant *Salmonella* Infections in Poultry. *Vet Sci*, 438.
- Kim W, B. S. (2011). Biochemical characterization of digestive enzymes in the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae) . *J Asia Pac Entomol.*, 14(1), 11-14.
- Kim, G., Lee, S., Levy Karin, E., Kim, H., Moriwaki, Y., Ovchinnikov, S., . . . Mirdita, M. (2025). Easy and accurate protein structure prediction using ColabFold. *Nature Protocols*, 620-642.
- Kim, M., Chen, Q., Echterhof, A., Pennetzdorfer, N., McBride, R., Banaei, N., . . . Bollyky, P. (2024). A blueprint for broadly effective bacteriophage-antibiotic cocktails against bacterial infections. *nature communications*, 9987.
- Kim, W., Bae, S., Park, K., Lee, S., Choi, Y., Han, S., & and Koh, Y. (2011). Biochemical characterization of digestive enzymes in the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). . *Journal of Asia-Pacific Entomology* , 14(1), 11-14.
- Klammsteiner, T., Walter, A., Bogataj, T., Heussler, C., Stres, B., Steiner, F., . . . Insam, H. (2020). The Core Gut Microbiome of Black Soldier Fly (*Hermetia illucens*) Larvae Raised on Low-Bioburden Diets. *Front. Microbiol.*
- Koehbach, J. (2017). Structure-activity relationships of insect defensins. *Frontiers in Chemistry*, 45.
- Koskella, B., & Brockhurst, M. (2014). Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS microbiology reviews*, 916-931.
- Kosznik-Kwaśnicka, K., Stasiłojć, M., Grabowski, Ł., Zdrojewska, K., Węgrzyn, G., & Węgrzyn, A. (2022). Efficacy and safety of phage therapy against *Salmonella enterica* serovars Typhimurium and Enteritidis estimated by using a battery of in vitro tests and the *Galleria mellonella* animal model. *Microbiological Research*, 127052.
- Lai, Y., & Gallo, R. (2009). AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends in immunology*, 131-141.

- Lalander, C. S., Gros Calvo, M., Ahrens, L., Josefsson, S., Wiberg, K., & Vinnerås, B. (2016). Fate of pharmaceuticals and pesticides in fly larvae composting. *Sci Total Environ*, 565, 279-286.
- Lalander, C., Diener, S., Zurbrugg, C., & Vinnerås, B. (2019). Effects of feedstock on larval development and process efficiency in waste treatment with black soldier fly (*Hermetia illucens*). *Journal of cleaner production*, 208, 211-219.
- Lalander, C., Ermolaev, E., Wiklicky, V., & Vinnerås, B. (2020). Process efficiency and ventilation requirement in black soldier fly larvae composting of substrates with high water content. *Science of The Total Environment*, 729, 138968.
- Lalander, C., Fidjeland, J., Diener, S., Eriksson, S., & Vinneras, B. (2015). High waste-to-biomass conversion and efficient *Salmonella* spp. reduction using black soldier fly for waste recycling. *Agronomy for Sustainable Development*, 35, 261–271.
- Lamberty, M., Ades, S., Uttenweiler-Joseph, S., Brookhart, G., Bushey, D., Hoffmann, J., & Bulet, P. (1999). Insect Immunity: isolation from the lepidopteran *Heliothis virescens* of a novel insect defensin with potent antifungal activity. *Journal of Biological Chemistry*, 9320-9326.
- Lavine, M., & Strand, M. (2002). Insect hemocytes and their role in immunity. *Insect Biochem Mol Biol*.
- Lee, E., Jeong, K., Lee, J., Shin, A., Kim, J., Lee, J., . . . Kim, Y. (2013). Structure-activity relationships of cecropin-like peptides and their interactions with phospholipid membrane. *BMB reports*, 282.
- Lehrer, R., & Ganz, T. (2002). Defensins of vertebrate animals. . *Current opinion in immunology*, 96-102.
- Leitner, L., Kessler, T., & McCallin, S. (2024). Innovations in Phage Therapy for Urinary Tract Infection. *European Urology Focus*, 722-725.
- Lemaitre, B. & (2007). The host defense of *Drosophila melanogaster*. *Annual Review of Immunology*, 697-743.
- Lemaitre, B., & Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. *Annu Rev Immunol.*, 697-743.
- Lemaitre, B., & Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. *Annual Review of Immunology*, 697-743.
- Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J., & Hoffmann, J. (1996). The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell*, 973-983.
- Lemaitre, B., Reichhart, J., & Hoffmann, J. (1997). *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proceedings of the National Academy of Sciences*, 14614-14619.
- Leong, S., Rahman, S., Kutty, M., Malakahmad, A., & Tan, C. (2016). Feasibility study of biodiesel production using lipids of *Hermetia illucens* larva fed with organic waste. *Waste Management*, 47, 84-90.
- Li, Q., Zheng, L., Qui, N., Cai, H., Tomberlin, J., & Yu, Z. (2011). Bioconversion of dairy manure by black soldier fly (Diptera: Stratiomyidae) for biodiesel and sugar production. *Waste Management*, 31, 1316-1320.
- Li, Z., Mao, R., Teng, D., Hao, Y., Chen, H., Wang, X., . . . Wang, J. (2017). Antibacterial and immunomodulatory activities of insect defensins-DLP2 and DLP4 against multidrug-resistant *Staphylococcus aureus*. *Scientific reports*, 7(1), 1-16.
- Li, Z., Mao, R., Teng, D., Hao, Y., Chen, H., Wang, X., . . . Wang, J. (2017). Antibacterial and immunomodulatory activities of insect defensins-DLP2 and DLP4 against multidrug-resistant *Staphylococcus aureus*. *Scientific reports*, 12124.

- Li, Z., Tan, S., Zheng, Y., Liang, H., Wang, Y., Zhang, Y., . . . Li, X. (2021). Insect antimicrobial peptides and their applications in agriculture and aquaculture. *Journal of Agricultural and Food Chemistry*, 1622-1633.
- Lim, S., Lee, L., & Wu, T. (2016). Sustainability of using composting and vermicomposting technologies for organic solid waste biotransformation: recent overview, greenhouse gases emissions and economic analysis. *Journal of Cleaner Production*, 111, 262-278.
- Lin, S., & Shelomi, M. (2024). Black Soldier Fly (*Hermetia illucens*) Microbiome and Microbe Interactions: A Scoping Review. *Animals* , 3183.
- Lindberg, H., McKean, K., & Wang, I. (2014). Phage fitness may help predict phage therapy efficacy. *Bacteriophage*, e964081.
- Lindsay, S., & Wasserman, S. (2014). Conventional and non-conventional *Drosophila* Toll signaling. *Developmental & Comparative Immunology*, 16-24.
- Lipsky, B. A., Holroyd, K. J., Zasloff, M., & Pollak, R. A. (2008). topical versus systemic antimicrobial therapy for treating mildly infected diabetic foot ulcers: a randomized, controlled, double-blinded, multicenter trial of pexiganan cream. *Clinical Infectious Diseases*, 1537-1545.
- Liu, C., Wang, C., & Yao, H. (2019). Comprehensive Resource Utilization of Waste Using the Black Soldier Fly (*Hermetia illucens* (L.)) (Diptera: Stratiomyidae). *Animals (Basel)*, 9(6), 349.
- Liu, L., Wei, Y., & Wang, D. (2021). Identification of core genes of Toll-like receptor pathway from *Lymantria dispar* and induced expression upon immune stimulant. *Insects*, 827.
- Liu, S., Raheel Tariq, M., Zhang, Q., Wang, H., Wang, F., Zheng, C., . . . and Wang, L. (2024). Dietary Influence on Growth, Physicochemical Stability, and Antimicrobial Mechanisms of Antimicrobial Peptides in Black Soldier Fly Larvae. *Insects*, 872.
- Liu, X., Chen, X., Wang, H., Yang, Q., ur Rehman, K., Li, W., & and Zheng, L. (2020). Dynamic changes of nutrient composition throughout the entire life cycle of black soldier fly. *PLoS ONE*, e0238375.
- Loc Carrillo, C., Atterbury, R., El-Shibiny, A., Connerton, P., Dillon, E., Scott, A., & Connerton, I. (2005). Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. . *Applied and environmental microbiology*, 6554-6563.
- Lomonaco, G., Franco, A., De Smet, J., Scieuzo, C., Salvia, R., & Falabella, P. (2024). Larval Frass of *Hermetia illucens* as Organic Fertilizer: Composition and Beneficial Effects on Different Crops. *Insects*.
- Lowenberger, C., Bulet, P., Charlet, M., Hetru, C., Hodgeman, B., Christensen, B., & Hoffmann, J. (1995). Insect immunity: isolation of three novel inducible antibacterial defensins from the vector mosquito, *Aedes aegypti*. *Insect biochemistry and molecular biology*, 867-873.
- Lu, J., Guo, Y., Muhmood, A., Zeng, B., Qiu, Y., Wang, P., & Ren, L. (2022). Probing the antioxidant activity of functional proteins and bioactive peptides in *Hermetia illucens* larvae fed with food wastes. *Scientific Reports*, 2799.
- Ma, J., Lei, Y., Rehman, K., Yu, Z., Zhang, J., Li, W., . . . and Zheng, L. (2018). Dynamic effects of initial pH of substrate on biological growth and metamorphosis of black soldier fly (Diptera: Stratiomyidae). *Environ Entomol*, 47(1), 159-165.
- Macharia, R., Ombura, F., & Aroko, E. (2015). Insects' RNA Profiling Reveals Absence of "Hidden Break" in 28S Ribosomal RNA Molecule of Onion Thrips, *Thrips tabaci*. *J Nucleic Acids*, 965294.

- MacKenzie, S., Roher, N., Boltaña, S., & Goetz, F. (2010). Peptidoglycan, not endotoxin, is the key mediator of cytokine gene expression induced in rainbow trout macrophages by crude LPS. *Molecular Immunology*, 1450-1457.
- Magalhães, R., Sánchez-López, A., Leal, R. S., Martínez-Llorens, S., Oliva-Teles, A., & Peres, H. (2017). Black soldier fly (*Hermetia illucens*) pre-pupae meal as a fishmeal replacement in diets for European seabass (*Dicentrarchus labrax*). *Aquaculture*, 79-85.
- Magana, M., Pushpanathan, M., Santos, A. L., Leanse, L., Fernandez, M., Ioannidis, A., . . . Tegos, G. P. (2020). The value of antimicrobial peptides in the age of resistance. *The Lancet Infectious Diseases*, e216-e230.
- Mahlapuu, M., Håkansson, J., Ringstad, L., & Björn, C. (2016). Antimicrobial peptides: an emerging category of therapeutic agents. *Frontiers in Cellular and Infection Microbiology*, 194.
- Mahlapuu, M., Håkansson, J., Ringstad, L., & Björn, C. (2016). Antimicrobial peptides: an emerging category of therapeutic agents. *Frontiers in cellular and infection microbiology*, 194.
- Mai, V., Ukhanova, M., Reinhard, M., Li, M., & Sulakvelidze, A. (2015). Bacteriophage administration significantly reduces *Shigella* colonization and shedding by *Shigella*-challenged mice without deleterious side effects and distortions in the gut microbiota. *Bacteriophage*, e1088124.
- Makkar, H., Tran, G., Heuzé, V., & Ankers, P. (2014). State-of-the-art on use of insects as animal feed. *Animal Feed Science Technology*, 1-33.
- Mannucci, A., Panariello, L., Abenaim, L., Coltelli, B. M., Ranieri, A., Conti, B., . . . Castagna, A. (2024). From Food Waste to Functional Biopolymers: Characterization of Chitin and Chitosan Produced from Prepupae of Black Soldier Fly Reared with Different Food Waste-Based Diets. *Foods*.
- Marston, H., Dixon, D., Knisely, J., Palmore, T., & Fauci, A. (2016). Antimicrobial resistance. *Jama*, 1193-1204.
- Mastoraki, M. K., I. T., G. K., & Mente, E. (2020). Nutritional evaluation of insect meals and their effects on growth performance and fish health: A review. *Aquaculture Research*, 4398–4412.
- Materazzi, A., Bottai, D., Campobasso, C., Klatt, A., Cesta, N., De Masi, M., . . . Di Luca, M. (2022). Phage-based control of methicillin resistant *Staphylococcus aureus* in a *Galleria mellonella* model of implant-associated infection. *International Journal of Molecular Sciences*, 14514.
- Mayo Clinic. (2025, 04 30). *Salmonella infection*. Retrieved from Salmonella Infection: <https://www.mayoclinic.org/diseases-conditions/salmonella/symptoms-causes/syc-20355329>
- McDonald, E., Jones, T., Plate, L., Meiler, J., & Gulsevin, A. (2023). Benchmarking AlphaFold2 on peptide structure prediction. *Structure*, 111-119.
- McKean, K., Yourth, C., Lazzaro, B., & Clark, A. (2008). The evolutionary costs of immunological maintenance and deployment. *BMC Evol Biol*.
- Medicines & Healthcare products Regulatory Agency. (2025). *Regulatory considerations for therapeutic use of bacteriophages in the UK*. Medicines & Healthcare products Regulatory Agency.
- Meneguz, M., Gasco, L., & Tomberlin, J. (2018). Impact of pH and feeding system on black soldier fly (*Hermetia illucens*, L; Diptera: stratiomyidae) larval development. *PLoS ONE*, 13(8).
- Ment, D., & Mishra, S. (2025). The integral role of the Black Soldier fly, *Hermetia illucens* L., microbiota in its life history. *Front. Microbiome*.

- Mirdita, M., Schütze, K., Moriwaki, Y., Heo, L., Ovchinnikov, S., & Steinegger, M. (2022). ColabFold: Making protein folding accessible to all. *Nature Methods*.
- Mookherjee, N., Anderson, M. A., Haagsman, H. P., & Davidson, D. J. (2020). Antimicrobial host defence peptides: functions and clinical potential. *Nature Reviews Drug Discovery*, 311-332.
- Moretta, A., Salvia, R., Scieuzo, C., Di Somma, A., Vogel, H., Pucci, P., . . . Falabella, P. (2020). A bioinformatic study of antimicrobial peptides identified in the Black Soldier Fly (BSF) *Hermetia illucens* (Diptera: Stratiomyidae). *Sci Rep*, 10(1).
- Moretta, A., Salvia, R., Scieuzo, C., Di Somma, A., Vogel, H., Pucci, P., . . . Falabella, P. (2020). A bioinformatic study of antimicrobial peptides identified in the Black Soldier Fly (BSF) *Hermetia illucens* (Diptera: Stratiomyidae). *Scientific reports*, 16875.
- Morpeth, S., Ramadhani, H., & Crump, J. (2009). Invasive non-typhi *Salmonella* disease in Africa. *Clinical infectious diseases*, 606-611.
- Mueller, O., Lightfoot, S., & Schroeder, A. (2004). RNA Integrity Number (RIN) – Standardization of RNA Quality Control. *RNA integrity number (RIN)–standardization of RNA quality control*, 1-8.
- Muliya Sankappa, N., Shivani Kallappa, G., Kallihosuru Boregowda, K., Mandrira Ramakrishna, N., Kattapuni Suresh, P., Shriraje Balakrishna, D., . . . Deshotel, M. (2024). Novel lytic bacteriophage AhFM11 as an effective therapy against hypervirulent *Aeromonas hydrophila*. *Scientific Reports*, 16882.
- Myers, H., Tomberlin, J., Lambert, B., & Kattes, D. (2008). Development of Black Soldier Fly (Diptera: Stratiomyidae) Larvae Fed Dairy Manure . *Environmental Entomology*, 37(1), 11–15.
- Myllymäki, H., Valanne, S., & Rämetsä, M. (2014). The *Drosophila* Imd Signaling Pathway. *he Journal of Immunology*, 3455–3462.
- Mylonakis, E., Podsiadlowski, L., Muhammed, M., & Vilcinskas, A. (2016). Diversity, evolution and medical applications of insect antimicrobial peptides. *Philosophical Transactions of the Royal Society B: Biological Sciences*.
- Nagib, M., Sayed, A., Korany, A., Abdelkader, K., Shari, F., Mackay, W., & Rateb, M. (2025). Human Defensins: Structure, function, and potential as therapeutic antimicrobial agents with highlights against SARS CoV-2. *Probiotics and Antimicrobial Proteins*, 1563-1583.
- Nakagaki, B., & Defoliart, G. (1991). Comparison of diets for mass-rearing *Acheta domesticus* (Orthoptera: Gryllidae) as a novelty food, and comparison of food conversion efficiency with values reported for livestock. *ournal of Economic Entomology*, 891-896.
- Nakamura, S., Ichiki, R., Shimoda, M., & Morioka, S. (2016). Small-scale rearing of the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae), in the laboratory: low-cost and year-round rearing. *Applied entomology and zoology*, 51(1), 161-166.
- Nakamura, S., Ichiki, R., Shimoda, M., & Morioka, S. (2016). Small-scale rearing of the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae), in the laboratory: low-cost and year-round rearing. *Applied Entomology and Zoology*, 51(1), 161-166.
- Nale, J., Vinner, G., Lopez, V., Thanki, A., Phothaworn, P., Thiennimitr, P., . . . Galyov, E. (2021). An optimized bacteriophage cocktail can effectively control *Salmonella* in vitro and in *Galleria mellonella*. *Frontiers in Microbiology*, 609955.
- Nascimento, E., Queiroz, E., Vidal, L., Junqueira, C., Souza, B., Manigat, D., . . . Albuquerque, É. (2025). Optimization of genomic DNA and total RNA extraction protocols from microlepidopterans for high-fidelity long-read sequencing. *BMC Methods*, 19.

- National Archives. (2025, 10 20). *Code Of Federal Regulations*. Retrieved from § 172.785 Listeria-specific bacteriophage preparation.: https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-172/subpart-H/section-172.785?utm_source=chatgpt.com
- Nguyen, E., Nemerow, G., & Smith, J. (2010). Direct evidence from single-cell analysis that human α -defensins block adenovirus uncoating to neutralize infection. *Journal of virology*, 4041-4049.
- Nguyen, T., Tomberlin, J., & VanLaerhoven, S. (2013). Influence of resources on *Hermetia illucens* (Diptera:Stratiomyidae) larval development. *Journal of Medical Entomology*, 50(4), 898-906.
- Nielsen, H., Engelbrecht, J., Brunak, S., & Von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein engineering*, 1-6.
- Nyakeri, E., Ayieko, M., Amimo, F., Salum, H., & Ogola, H. (2019). An optimal feeding strategy for black soldier fly larvae biomass production and faecal sludge reduction. *Journal of Insects as Food and Feed*, 5(3), 201-213.
- Ogino, K., Eda-Fujiwara, H., Fujiwara, H., & Ishikawa, H. (1990). What causes the aphid 28S rRNA to lack the hidden break? *Journal of Molecular Evolution*, 509-513.
- Omar, R., Natasha, N., & Edward, G. (2025). Regulatory Landscape and the Potential of Bacteriophage Applications in the United States' Food Industry. *Regulatory Landscape and the Potential of Bacteriophage Applications in the United States' Food Industry*, 100510.
- O'Neill, J. (2014). Antimicrobial resistance: tackling a crisis for the health and wealth of nations. *Rev. Antimicrob. Resist.*
- Oonincx, D., Van Itterbeeck, J., Heetkamp, M., Van Den Brand, H., Van Loon, J., & Van Huis, A. (2010). An exploration on greenhouse gas and ammonia production by insect species suitable for animal or human consumption. *PloS one*, 5(12), e14445.
- Oonincx, D., Volk, N., Diehl, J., Van Loon, J., & Belušič, G. (2016). Photoreceptor spectral sensitivity of the compound eyes of black soldier fly (*Hermetia illucens*) informing the design of LED-based illumination to enhance indoor reproduction. *Journal of Insect Physiology*, 95, 133-139.
- Osta, A. M., Christophides, K. G., Vlachou, D., & Kafatos, C. F. (2004). Innate immunity in the malaria vector *Anopheles gambiae*: comparative and functional genomics. *Journal of Experimental Biology*.
- Padhi, B., Singh, M., Rosales, M., Pelletier, G., & Cakmak, S. (2018). A PCR-based quantitative assay for the evaluation of mRNA integrity in rat samples. *Biomol Detect Quantif*, 18-23.
- Palladino, R., Cigliero, S., Bonin, S., Pitacco, P., Nicolin, V., Previderè, C., . . . Fattorini, P. (2015). Reliability of RT-qPCR from degraded RNA samples: An in vitro model. *Forensic Science International*, 60-62.
- Palma, L., Ceballos, S., Johnson, P., Niemeier, D., Pitesky, M., & VanderGheynst, J. (2018). Cultivation of black soldier fly larvae on almond byproducts: impacts of aeration and moisture on larvae growth and composition. *Journal of the Science of Food and Agriculture*, 98(15), 5893-5900.
- Pålsson-McDermott, E., & O'Neill, L. (2007). Pålsson-McDermott, E.M. and O'Neill, L.A., . *Biochemical Society Transactions*, 1437-1444.
- Pan, X., Zhou, G., Wu, J., Bian, G., Lu, P., Raikhel, A., & Zi, Z. (2012). *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti*. *PNAS*, 23-31.

- Park, H. (2016). *Black Soldier Fly Larvae Manua*. Retrieved 07 26, 2022, from https://scholarworks.umass.edu/cgi/viewcontent.cgi?article=1015&context=sustainableumass_studentshows
- Park, S., & Yoe, S. (2017). A novel cecropin-like peptide from black soldier fly, *Hermetia illucens*: Isolation, structural and functional characterization. *Entomological Research*, 47(2), 115-124.
- Park, S., Kim, J., & Yoe, S. (2015). Purification and characterization of a novel antibacterial peptide from black soldier fly (*Hermetia illucens*) larvae. *Developmental & Comparative Immunology*, 52(1).
- Park, W. (2020). Coiled-Coils: The Molecular Zippers that Self-Assemble Protein Nanostructures. *International journal of molecular sciences*, 3584.
- Parodi, A., de Boer, I., Slegers, P., & van Zanten, H. (2024). Scaling up black soldier fly farming to reduce food waste: Assessing environmental and land use trade-offs'. *Nature Food*, 22-31.
- Patli, Y. (2011). Toll-like receptors in bony fish: from genomics to function. *Dev Comp Immunol.*, 1263-1272.
- Peng, J., Li, L., Wan, Y., Yang, Y., An, X., Yuan, K., . . . Liang, G. (2024). Molecular characterization and antimicrobial activity of cecropin family in *Hermetia illucens*. *Developmental & Comparative Immunology*, 105111.
- Perednia, D., Anderson, J., & Rice, A. (2017). A Comparison of the Greenhouse Gas Production of Black Soldier Fly Larvae versus Aerobic Microbial Decomposition of an Organic Feed Material. *Research & Reviews: Journal of Ecology and Environmental Sciences*.
- Perma, A. D., Julita, U., F, L. L., & Putra, R. E. (2020). Mating Success and Reproductive Behavior of Black Soldier Fly *Hermetia illucens* L. (Diptera, Stratiomyidae) in Tropics. *Journal of Entomology*, 5(2).
- Pimentel, D., & Pimentel, M. (2003). Sustainability of meat-based and plant-based diets and the environment. *The American Journal of Clinical Nutrition*, 78(3), 660S–663S.
- Pirnay, J., & Verbeken, G. (2023). Magistral Phage Preparations: Is This the Model for Everyone? *Clinical Infectious Diseases*, S360-S369.
- Popa, G., & Papa, M. (2021). Salmonella spp. infection - a continuous threat worldwide. *Germes*, 88.
- Poudel, A., Zhu, S., Cooper, N., Little, P., Tarrant, C., Hickman, M., & Yao, G. (2023). The economic burden of antibiotic resistance: A systematic review and meta-analysis. *Plos one*, 0285170.
- Qi, S., Gao, B., & Zhu, S. (2021). Molecular Diversity and Evolution of Antimicrobial Peptides in *Musca domestica*. *Diversity*, 107.
- Quispe-Villegas, G., Alcántara-Lozano, G., Cuicapuza, D., Laureano, R., Ayzanoa, B., Tsukayama, P., & Tamariz, J. (2025). In vivo evaluation of phage therapy against *Klebsiella pneumoniae* using the *Galleria mellonella* model and molecular characterization of a novel Drulivirus phage species. *Microbiol Spectr*, e01145-24.
- Rai, S., & Kumar, A. (2022). Bacteriophage therapeutics to confront multidrug-resistant *Acinetobacter baumannii*-a global health menace. *Environmental microbiology reports*, 347-364.
- Rajanbabu, V., & Chen, J. (2011). pplications of antimicrobial peptides from fish and perspectives for the future. *Peptides*, 415-420.
- Ramirez, L. J., & Dimopoulos, G. (2010). The Toll immune signaling pathway control conserved anti-dengue defenses across diverse *Ae. aegypti* strains and against multiple dengue virus serotypes. *Dev Comp Immunol*, 625-629.

- Redrejo-Rodríguez, M., & García, P. (2018). "FAGOMA: Spanish Network of Bacteriophages and Transducer Elements"-V Meeting Report. *MDPI*.
- Rehman, U. K., Hollah, C., Wiesotzki, K., Rehman, U. R., Rehman, U. A., Zhang, J., . . . Aganovic, K. (2022). Black soldier fly, *Hermetia illucens* as a potential innovative and environmentally friendly tool for organic waste management: A mini-review. *Waste Manag Res.*, 81-97.
- Remacle, A., Shiryaev, S., Oh, E., Cieplak, P., Srinivasan, A., Wei, G., . . . Day, R. (2008). Substrate cleavage analysis of furin and related proprotein convertases: a comparative study. *Journal of Biological Chemistry*, 20897-20906.
- Research and Markets. (2022). *Black Soldier Fly Market by Product, by Application, and Geography - Global Forecast to 2033*. Retrieved 08 09, 2022, from <https://www.researchandmarkets.com/reports/4986109/black-soldier-fly-market-by-product-by>
- Restaino, L., Grauman, G., McCall, W., & Hill, W. (1977). Effects of varying concentrations of novobiocin incorporated into two *Salmonella* plating media on the recovery of four Enterobacteriaceae. *Applied and Environmental Microbiology*, 585-589.
- Robert, X., & Gouet, P. (2014). Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Research*, W320–W324.
- Romero, I., Ruvinsky, I., & Yoac, G. (2014). Comparative studies of gene expression and the evolution of gene regulation. *Nat Rev Genet*, 505-516.
- Rudrapatna, V. A. (2012). *Drosophila* cancer models. *Dev. Dyn.*, 107-118.
- Ryckebusch, F., Tian, Y., Rapin, M., Schüpfer, F., Hanson, M., & Lemaitre, B. (2025). Layers of immunity: Deconstructing the *Drosophila* effector response. *bioRxiv*, 2025-03.
- Sánchez-Muros, M., Barroso, F., & Manzano-Agugliaro, F. (2014). Insect meal as renewable source of food for animal feeding: a review. *Journal of Cleaner Production*, 65, 16-27.
- Sackton, B. T., Lazzaro, P. B., Schlenke, A. T., Evans, D. J., Hultmark, D., & Clark, G. A. (2007). Dynamic evolution of the innate immune system in *Drosophila*. *nature genetics*, 1461-1468.
- Salam, M., Al-Amin, M., Salam, M., Pawar, J., Akhter, N., Rabaan, A., & Alqumber, M. (2023). Antimicrobial resistance: a growing serious threat for global public health. *In Healthcare*, 1946.
- Salomone, R., Saija, G., Mondello, G., Giannetto, A., Fasulo, S., & Savastano, D. (2017). Environmental impact of food waste bioconversion by insects: Application of Life Cycle Assessment to process using *Hermetia illucens*. *Journal of Cleaner Production*, 140, 890-905.
- Salzman, N. (2010). Paneth cell defensins and the regulation of the microbiome: detente at mucosal surfaces. *Gut microbes*, 401-406.
- Sanches-Fernandes, G., Sá-Correia, I., & Costa, R. (2022). Vibriosis Outbreaks in Aquaculture: Addressing Environmental and Public Health Concerns and Preventive Therapies Using Gilthead Seabream Farming as a Model System. *Frontiers in microbiology*, 904815.
- Sato, H., & Feix, J. (2006). Peptide–membrane interactions and mechanisms of membrane destruction by amphipathic α -helical antimicrobial peptides. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1245-1256.
- Satyavathi, V., Minz, A., & Nagaraju, J. (2014). Nodulation: an unexplored cellular defense mechanism in insects. *Cell Signal*, 1753-63.

- Schiavone, A., De Marco, M., & Gai, F. (2023). Insects as sustainable protein ingredients in animal feed: Nutritional and health effects. *Animal Feed Science and Technology*, 115569.
- Schooley, R., Biswas, B., Gill, J., Hernandez-Morales, A., Lancaster, J., Lessor, L., . . . Segall, A. (2017). Development and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a Disseminated Resistant *Acinetobacter baumannii* Infection. *Antimicrobial agents and chemotherapy*, 10-1128.
- Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors – occurrence, properties and removal. *Journal of applied microbiology*, 1014-1026.
- Schwenke, R., Lazzaro, B., & Wolfner, M. (2016). Reproduction-Immunity Trade-Offs in Insects. *Annu Rev Entomol.*, 239-56.
- Seed, D. K., & Dennis, J. J. (2009). Experimental Bacteriophage Therapy Increases Survival of *Galleria mellonella* Larvae Infected with Clinically Relevant Strains of the *Burkholderia cepacia* Complex. *Antimicrob Agents Chemother*, 2205–2208.
- Selsted, M., Brown, D., DeLange, R., & Lehrer, R. (1983). Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages. *Journal of Biological Chemistry*, 14485-14489.
- Setti, L., Francia, E., Pulvirenti, A., Gigliano, S., Zaccardelli, M., Pane, C., . . . Ronga, D. (2019). Use of black soldier fly (*Hermetia illucens* (L.), Diptera: Stratiomyidae) larvae processing residue in peat-based growing media. *Waste Management*, 95, 278-288.
- Shafee, T., Lay, F., Phan, T., Anderson, M., & Hulett, M. (2017). Convergent evolution of defensin sequence, structure and function. *Cellular and Molecular Life Sciences*, 663-682.
- Shah, P. N., Maistrou, S., Willemsen, I., Van Loon, J., & Dicke, M. (2024). Transcriptomic response of *Hermetia illucens* L. (Diptera: Stratiomyidae) to wounding and Gram-negative bacterial infection. *Transcriptomic response of Hermetia illucens L. (Diptera: Stratiomyidae) to wounding and Gram-negative bacterial infection*, 751-771.
- Shah, P., Ruan, X., van Loon, J., & Dicke, M. (2023). Temperature-modulated host-pathogen interactions between *Hermetia illucens* L. (Diptera: Stratiomyidae) and *Pseudomonas protegens* Pf-5. *Journal of Invertebrate Pathology*, 107934.
- Sharma, D., Singh, I., Sharma, J., Verma, I., & Ratn, A. (2025). Bacteriophage Therapy to Combat Microbial Infections and Antimicrobial Resistance. *Journal of Basic Microbiology*, e70090.
- She, W., Xiao, Q., Meng, Y., Zhao, P., Wu, C., Huang, F., . . . Peng, D. (2023). Isolated and identified pathogenic bacteria from black soldier fly larvae with “soft rot” reared in mass production facilities and its incidence characteristics. *Waste Management*, 85-95.
- Sheppard, D. C., Newton, G. L., Thompson, S. A., & Savage, S. (1994). A value-added manure management-system using the Black Soldier Fly. *Bioresource Technology*, 50(3), 275-279.
- Sheppard, D. C., Tomberlin, J. K., Joyce, J. A., Kiser, B. C., & Sumner, S. M. (2002). Rearing methods for the black soldier fly (Diptera: stratiomyidae). *Journal of Medical Entomology*, 39(4), 695-698.
- Sievers, F. &. (2014). Clustal Omega, accurate alignment of very large numbers of sequences. *Methods in Molecular Biology*, 105–116.
- Silvaraju, S., Zhang, Q., Kittelmann, S., & Puniamoorthy, N. (2024). Genetics, age, and diet influence gut bacterial communities and performance of black soldier fly larvae (*Hermetia illucens*). *anim microbiome*, 56.

- Singh, B., Rohit, Manju, K., Sharma, R., Bhushan, B., Ghosh, S., & Goel, G. (2023). Nano-Conjugated Food-Derived Antimicrobial Peptides As Natural Biopreservatives: A Review of Technology and Applications. *Antibiotics (Basel)*, 244.
- Smith, H. W., & Huggins, M. B. (1982). Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. *J. Gen. Microbiol.*, 307-318.
- Smith, H. W., Huggins, M. B., & Shaw, K. M. (1987). The control of experimental *Escherichia coli* diarrhoea in calves by means of bacteriophages. *J. Gen. Microbiol.*, 1111-1126.
- Soffer, N., Woolston, J., Li, M., Das, C., & Sulakvelidze, A. (2017). Bacteriophage preparation lytic for *Shigella* significantly reduces *Shigella sonnei* contamination in various foods. *PLoS One*, e0175256.
- Souza-Neto, A. J., Sim, S., & Dimopoulos, G. (2009). An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *PNAS*, 17841-17846.
- Sprangers, T. O. (2017). Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. *Journal of the Science of Food and Agriculture*, 2594–2600.
- Stark, R., Grzelak, M., & Hadfield, J. (2019). RNA sequencing: the teenage years. *Nature Reviews Genetics*, 631-656.
- Stec, W., Vidal, O., & Zeidler, P. M. (2013). *Drosophila* SOCS36E negatively regulates JAK/STAT pathway signaling via two separable mechanisms. *Mol Biol Cell*, 3000–3009.
- Steiner, H., Hultmark, D., Engström, Å., Bennich, H., & Boman, H. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature*, 246-248.
- Stoven, S., Silverman, N., Junell, A., Hedengren-Olcott, M., Erturk, D., Engstrom, Y., . . . Hultmark, D. (2003). Caspase-mediated processing of the *Drosophila* NF- κ B factor Relish. *PNAS*, 5991-5996.
- Stowell, N., Seideman, J., Raymond, H., Smalley, K., Lamb, R., Egenolf, D., . . . Flavell, R. (2009). Long-term activation of TLR3 by poly (I: C) induces inflammation and impairs lung function in mice. *Respiratory research*, 43.
- Strand, M. (2008). The insect cellular immune response. *Insect Science*.
- Subramanian, A. (2024). Emerging roles of bacteriophage-based therapeutics in combating antibiotic resistance. *Frontiers in Microbiology*, 1384164.
- Surendra, K., Tomberlin, J., van Huis, A., Cammack, J., Heckmann, L., & and Khanal, S. (2020). Rethinking organic wastes bioconversion: Evaluating the potential of the black soldier fly (*Hermetia illucens* (L.)) (Diptera: Stratiomyidae) (BSF). *Waste Management*, 117, 58-80.
- Sutton, J., & Pritts, T. (2014). Human beta-defensin 3: A novel inhibitor of *Staphylococcus*-Produced biofilm production. Commentary on “Human β -defensin 3 inhibits antibiotic-resistant *Staphylococcus* biofilm formation”. *Journal of Surgical Research*, 99-100.
- Takahashi, M., Umehara, Y., Yue, H., Trujillo-Paez, J., Peng, G., Nguyen, H., . . . Niyonsaba, F. (2021). The antimicrobial peptide human β -defensin-3 accelerates wound healing by promoting angiogenesis, cell migration, and proliferation through the FGFR/JAK2/STAT3 signaling pathway. *Frontiers in Immunology*, 712781.
- Takeda, K., Uehara, T., Liu, C., Kobayashi, T., Shimoda, M., & Yokoi, K. (2025). Genome sequence data and integrative RNA-Seq data of Black Soldier Fly (*Hermetia illucens*)(A Japanese strain). *bioRxiv*, 2025-07.
- Tanga, C., Waweru, J., Tola, Y., Onyoni, A., Khamis, F., Ekesi, S., & Paredes, J. (2021). Organic Waste Substrates Induce Important Shifts in Gut Microbiota of Black Soldier

- Fly (*Hermetia illucens* L.): Coexistence of Conserved, Variable, and Potential Pathogenic Microbes. *Front Microbiol.*
- Thanki, A., Brown, N., Millard, A., & Clokie, M. (2019). Genomic characterization of jumbo Salmonella phages that effectively target United Kingdom pig-associated Salmonella serotypes. *Frontiers in microbiology*, 1491.
- Thanki, A., Brown, N., Millard, A., & Clokie, M. (2019). Genomic characterization of jumbo Salmonella phages that effectively target United Kingdom pig-associated Salmonella serotypes. *Frontiers in microbiology*, 1491.
- Thanki, A., Clavijo, V., Healy, K., Wilkinson, R., Sicheritz-Pontén, T., Millard, A., & Clokie, M. (2022). Development of a Phage Cocktail to Target Salmonella Strains Associated with Swine. *Pharmaceutics (Basel)*, 58.
- Thanki, A., Hooton, S., Whenham, N., Salter, M., Bedford, M., O'Neill, H., & Clokie, M. (2023). A bacteriophage cocktail delivered in feed significantly reduced Salmonella colonization in challenged broiler chickens. *Emerg Microbes Infect*, 2217947.
- Thanki, A., Mignard, G., Atterbury, R., Barrow, P., Millard, A., & Clokie, M. (2022). Prophylactic Delivery of a Bacteriophage Cocktail in Feed Significantly Reduces Salmonella Colonization in Pigs. *Microbiology Spectrum*, e00422-22.
- Thiermann, W. (2024, December 17). *Can Black Soldier Fly Larvae (BSFL) producers displace fishmeal? Rethink Priorities*. Retrieved from Rethink Priorities: <https://rethinkpriorities.org/research-area/can-black-soldier-fly-larvae-bsfl-producers-displace-fishmeal/>
- Thomma, B., Cammue, B., & Thevissen, K. (2002). Plant defensins. *Planta*, 193-202.
- Tingle, F., Mitchell, E., & Copeland, W. (1975). The soldier fly, *Hermetia illucens* in poultry houses in north central Florida [Insect pests]. *Journal - Georgia Entomological Society*, 10(2), 179-183.
- Tingvall, T., Roos, E., & Engström, Y. (2001). The imd gene is required for local Cecropin expression in *Drosophila* barrier epithelia. *EMBO reports*.
- Tippayadara, N., Dawood, M., Krutmuang, P., Hoseinifar, S., Doan, H., & Paolucci, M. (2021). Replacement of Fish Meal by Black Soldier Fly (*Hermetia illucens*) Larvae Meal: Effects on Growth, Haematology, and Skin Mucus Immunity of Nile Tilapia, *Oreochromis niloticus*. *Animals (Basel)*.
- Tomberlin, J. K., & Sheppard, D. C. (2002). Factors Influencing Mating and Oviposition of Black Soldier Flies (Diptera: Stratiomyidae) in a Colony. *Journal of Entomological Science*, 37(4), 345-352.
- Tomberlin, J. K., Sheppard, D. C., & Joyce, J. A. (2002). Selected Life-History Traits of Black Soldier Flies (Diptera: Stratiomyidae) Reared on Three Artificial Diets. *Annals of the Entomological Society of America*, 95(3), 379–386.
- Tomberlin, J., Adler, P., & Myers, H. (2009). Development of the black soldier fly (Diptera: stratiomyidae) in relation to temperature. *Environmental Entomology*, 38(3), 930-934.
- Twort, F. (1915). An investigation on the nature of ultra-microscopic viruses. *Acta Kravsi*.
- Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J. M., Lemaitre, B., . . . Imler, J. L. (2000). Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. *Immunity*, 737-748.
- UMC Utrecht. (2025). First experimental therapy at UMC Utrecht with personalized bacteriophages. *UMC Utrecht*.
- United Nations. (1997).) Survey of mayors: major urban problems. In: 'Governance for Sustainable Growth and Equity', Report of the International Conference,. New York: UNDP.

- United Nations. (2017). *World Population Prospects*. Retrieved 07 26, 2022, from https://population.un.org/wpp/publications/files/wpp2017_keyfindings.pdf
- University of Nebraska Medical Centre. (2025, 07 21). *AMP Database Search*. Retrieved from APD6 Antimicrobial Peptide Database: https://aps.unmc.edu/?utm_source=chatgpt.com
- Valanne, S., Wang, J., & Rämet, M. (2011). The *Drosophila* toll signaling pathway. *The Journal of Immunology*, 649-656.
- van Broekhoven, S. O. (2015). Growth performance and feed conversion efficiency of three edible mealworm species (Coleoptera: Tenebrionidae) on diets composed of organic by-products. *Journal of Insect Physiology*, 1-10.
- Van Huis, A. (2021). Insects as food and feed: A new emerging agricultural sector. *Journal of Insects as Food and Feed*, 693–700.
- van Huis, A., & Oonincx, D. (2017). The environmental sustainability of insects as food and feed. A review. *Agronomy for Sustainable Development*, 37-43.
- Van Moll, L., De Smet, J., Paas, A., Tegtmeier, D., Vilcinskas, A., Cos, P., & Van Campenhout, L. (2022). In Vitro Evaluation of Antimicrobial Peptides from the Black Soldier Fly (*Hermetia illucens*) Against a Selection of Human Pathogens. *Microbiology Spectrum*, e01664-21.
- Van Moll, L., De Smet, J., Paas, A., Tegtmeier, D., Vilcinskas, A., Cos, P., & Van Campenhout, L. (2022). In Vitro Evaluation of Antimicrobial Peptides from the Black Soldier Fly (*Hermetia Illucens*) against a Selection of Human Pathogens. *Microbiol Spectr.*, 10(1).
- Vandeweyer, D., Bruno, D., Bonelli, M., IJdema, F., Lievens, B., Crauwels, S., . . . De Smet, J. (2023). Bacterial biota composition in gut regions of black soldier fly larvae reared on industrial residual streams: revealing community dynamics along its intestinal tract. *Front Microbiol.*
- Van-Huis, A. (2013). Potential of insects as food and feed in assuring food security. *Annual Review Entomology*, 563-583.
- Veltri, D., Kamath, U., & Shehu, A. (2018). Deep learning improves antimicrobial peptide recognition. *Bioinformatics*, 2740-2747.
- Vikram, A., Callahan, M., Woolston, J., Sharma, M., & Sulakvelidze, A. (2022). Phage biocontrol for reducing bacterial foodborne pathogens in produce and other foods. *Current Opinion in Biotechnology*, 102805.
- Vikram, A., Tokman, I. J., Woolston, J., & Sulakvelidze, A. (2020). Phage Biocontrol Improves Food Safety by Significantly Reducing the Level and Prevalence of *Escherichia coli* O157:H7 in Various Foods. *Journal of Food Protection*, 668-676.
- Vilcinskas, A., Mukherjee, K., & Vogel, H. (2013). Expansion of the antimicrobial peptide repertoire in the invasive ladybird *Harmonia axyridis*. *Proceedings of the Royal Society B: Biological Sciences*, 280(1750), 20122113.
- Vinodkumar, C., Neelagund, Y., & Kalsurmath, S. (2005). Bacteriophage in the treatment of experimental septicemic mice from a clinical isolate of multidrug resistant *Klebsiella pneumoniae*. *The Journal of communicable diseases*, 18-29.
- Vique, G., Mendoza-Barberá, E., Ramos-Barbero, M., Blanco-Picazo, P., Sala-Comorera, L., Quirós, P., . . . Rodríguez-Rubio, L. (2025). Efficacy of *Erwinia amylovora* and *Xanthomonas campestris* pv *campestris* phages to control fire blight and black rot in vivo. *Microbiology Spectrum*, e00280-25.
- Vogel, H., Mřller, A., Heckel, D., Gutzeit, H., & Vilcinskas, A. (2018). Nutritional immunology: diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*. *Developmental & Comparative Immunology*, 78, 141-148.

- Vogel, H., Müller, A., Heckel, D. G., Gutzeit, H., & Vilcinskas, A. (2018). Transcriptome analysis reveals high antimicrobial peptide diversity in the invasive ladybird *Harmonia axyridis*. *Ecology and Evolution*, 2844-2855.
- Vogel, H., Müller, A., Heckel, D., Gutzeit, H., & Vilcinskas, A. (2018). Nutritional immunology: Diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*. *Developmental & Comparative Immunology*, 141-148.
- Vogel, H., Müller, A., Heckel, D., Gutzeit, H., & Vilcinskas, A. (2018). Nutritional immunology: Diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*. *Dev Comp Immunol*, 141-148.
- Vogel, H., Muller, A., Heckel, G. D., Gutzeit, H., & Vilcinskas, A. (2018). Nutritional immunology: Diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*. *Elsevier*, 141-148.
- Vogel, H., Muller, A., Heckel, G. D., Gutzeit, H., & Vilcinskas, A. (2018). Nutritional immunology: Diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*. *Elsevier*, 141-148.
- Wagh, R., Priyadarshi, R., & Rhim, J. (2023). Novel insights into phage-based films and coatings: Innovative solutions for active food packaging systems. *Elsevier*, 609.
- Wang, G., Ran, J., Jia, C., Mohamed, A., Paredes-Montero, J., Al-Akeel, R., . . . Eleftherianos, I. (2025). Developmental transcriptomics reveals stage-specific immune gene expression profiles in *Spodoptera frugiperda*. *scientific reports*, 25044.
- Wang, M., Deng, Z., Li, Y., Xu, K., Ma, Y., Yang, S., & Wang, J. (2022). Antibiofilm property and multiple action of peptide PEW300 against *Pseudomonas aeruginosa*. *Frontiers in Microbiology*, 963292.
- Wang, Y., & Shelomi, M. (2017). Review of Black Soldier Fly (*Hermetia illucens*) as animal feed and human food. *Foods*, 6(10).
- Wang, Y., & Shelomi, M. (2017). Review of Black Soldier Fly (*Hermetia illucens*) as Animal Feed and Human Food. *Foods*, 6(10).
- Wang, Y., & Shelomi, M. (2017). Review of Black Soldier Fly (*Hermetia illucens*) as Animal Feed and Human Food. *Foods*, 6(10).
- Wang, Y., Zhang, Y., Wang, J., Kang, C., Hu, G., Guo, Y., . . . Wang, Y. (2023). Temperature dependent development of black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) from Yangtze River Delta region of China. *Elsevier*, 102163.
- Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics*, 57-63.
- WHO. (2025). Global antibiotic resistance surveillance report 2025. *WHO*.
- Willy, C. B., Classen, A., Deng, L., Dückting, A., Gross, J., Hammerl, J., . . . Rohde, C. (2023). Phage Therapy in Germany—Update 2023. *Phage therapy in Germany—update 2023*, 588.
- Wimley, W., & Hristova, K. (2011). Antimicrobial peptides: successes, challenges and unanswered questions. *The Journal of membrane biology*, 27-34.
- Winnebeck, E., Millar, C., & Warman, G. (2010). Why does insect RNA look degraded? *Journal of Insect Science*, 159.
- Wittebole, X., De Roock, S., & Opal, S. (2014). A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virulence*, 226-235.
- Wong, C., Ho, Y., Lim, J., Show, P., Chong, S., Chan, Y., . . . and Pan, G. (2020). In-situ yeast fermentation medium in fortifying protein and lipid accumulations in the harvested larval biomass of black soldier fly. *Processes*, 8(3), 337.

- Wong, C., Mohd Aris, M., Daud, H., Lam, M., Yong, C., Abu Hasan, H., . . . and Goh, P. (2020). In-Situ Yeast Fermentation to Enhance Bioconversion of Coconut Endosperm Waste into Larval Biomass of *Hermetia illucens*: Statistical Augmentation of Larval Lipid Content. *Sustainability*, 12(4), 1558.
- World Health Organization. (2018). *Salmonella (non-typhoidal)*. WHO.
- World Health Organization. (2025). *Global antibiotic resistance surveillance report 2025*. Global Report: WHO.
- Wu, Q., Patocka, J., & Kuca, K. (2018). Insect Antimicrobial Peptides, a Mini Review. *MDPI*.
- Xi, Z., Ramirez, J., & Dimopoulos, G. (2008). The *Aedes aegypti* toll pathway controls dengue virus infection. *PLoS Pathog*, 1000098.
- Xiao, X., Jin, L., Li, Y., Zhang, Y., & Liu, Q. (2022). Effects of different substrates on BSFL growth and safety. *Waste Management*, 72-81.
- Xu, D., & Lu, W. (2020). Defensins: A Double-Edged Sword in Host Immunity. *Sec. Microbial Immunology*, 764.
- Xu, Q., Wu, A., Zeng, X., & An, X. (2020). Identification and Expression Profiling of Chemosensory Genes in *Hermetia illucens* via a Transcriptomic Analysis. *Front Physiol*, 720.
- Xu, W., Zhang, L., Wang, J., Huang, Y., Zhang, Y., & Guo, Y. (2020). Identification and expression profiling of chemosensory genes in the antennae of the black soldier fly. *Insect Science*, 565-576.
- Xuan, J., Feng, W., Wang, J., Wang, R., Zhang, B., Bo, L., . . . Sun, L. (2023). Antimicrobial peptides for combating drug-resistant bacterial infections. *Drug Resistance Updates*, 100954.
- Yang, D., Chertov, O., Bykovskaia, S., Chen, Q., Buffo, M., Shogan, J., . . . Oppenheim, J. (1999). β -defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science*, 525-528.
- Yang, H., Kronhamn, J., Ekstrom, J., Korkut, G. G., & Hultmark, D. (2015). JAK/STAT signaling in *Drosophila* muscles controls the cellular immune response against parasitoid infection. *Embro Reports*, 1664-1672.
- Yen, M., Cairns, L., & Camilli, A. (2017). A cocktail of three virulent bacteriophages prevents *Vibrio cholerae* infection in animal models. *Nat Commun*, 14187.
- Yu, K., Lo, J., Yan, M., Yang, X., Brooks, D., Hancock, R., . . . Kizhakkedathu, J. (2017). nti-adhesive antimicrobial peptide coating prevents catheter associated infection in a mouse urinary infection model. *Biomaterials*, 69-81.
- Yu, S., Luo, F., Xu, Y., Zhang, Y., & Jin, L. (2022). *Drosophila* Innate Immunity Involves Multiple Signaling Pathways and Coordinated Communication Between Different Tissues. *Fromt Immunol*.
- Yuan, M., & Hasan, H. (2022). Effect of Feeding Rate on Growth Performance and Waste Reduction Efficiency of Black Soldier Fly Larvae (Diptera: Stratiomyidae). *Tropical Life Sciences Research*, 33(1), 179.
- Zabulione, A., Salaseviciene, A., Makstutiene, N., & Sarkinas, A. (2023). Exploring the Antimicrobial Potential and Stability of Black Soldier Fly (*Hermentia illucens*) Larvae Fat for Enhanced Food Shelf-Life. *Gels*.
- Zdybicka-Barabas, A., Stączek, S., Kunat-Budzyńska, M., & Cytryńska, M. (2025). Innate Immunity in Insects: The Lights and Shadows of Phenoloxidase System Activation. *Int J Mol Sci*.
- Zeng, C., Qi, L., Guan, C., Chang, Y., He, Y., Zhao, H., . . . Zhong, G. (2025). Phage Therapy Enhances Survival, Immune Response, and Metabolic Resilience in Pacific

- White Shrimp (*Litopenaeus vannamei*) Challenged with *Vibrio parahaemolyticus*. *Fishes*, 366.
- Zhan, S., Fang, G., Cai, M., Kou, Z., Xu, J., Cao, Y., . . . Xu, J. (2020). Genomic landscape and genetic manipulation of the black soldier fly *Hermetia illucens*, a natural waste recycler. *Cell research*, 50-60.
- Zhang, B., Yang, R., He, S., Dai, S., Hu, Q., Li, X., . . . Wu, D. (2024). Swill and Pig Manure Substrates Differentially Affected Transcriptome and Metabolome of the Black Soldier Fly Larvae. *Int J Mol Sci*, 12147.
- Zhang, J., Huang, L., He, J., Tomberlin, J., Li, J., Lei, C., . . . Yu, Z. (2010). An artificial light source influences mating and oviposition of black soldier flies, *Hermetia illucens*. *Insect Sci.* , 10(1), 202.
- Zhang, X., Wang, S., Li, T., Zhang, Q., Zhang, R., & Zhang, Z. (2021). Bacteriophage: A Useful Tool for Studying Gut Bacteria Function of Housefly Larvae, *Musca domestica*. *Microbiology Spectrum*, 10-1128.
- Zhang, Y., Chen, W., Wang, B., Rehman, K., Van Huis, A., Henawy, A., . . . Zhang, J. (2025). Enhancing Salmonella Inhibition in Black Soldier Fly Larvae (*Hermetia illucens* L.) Conversion by Bioaugmentation With Gut Microbiota. *Microbial Biotechnology*, 70242.
- Zhang, Z., Shen, J., Wang, H., & al, e. (2014). Attenuation of veterinary antibiotics in full-scale vermicomposting of swine manure via the housefly larvae (*Musca domestica*). *Sci Rep*, 4.
- Zhao, B., Lin, H., Yang, D., Ye, X., & Li, Z. (2016). Disulfide bridges in defensins. *Current topics in medicinal chemistry*, 206-219.
- Zhao, J., Wang, Y., Li, X., & Gai, Z. (2020). Genome-wide identification and characterization of Toll-like receptors (TLRs) in housefly (*Musca domestica*) and their roles in the insecticide resistance. *Int J Biol Macromol.*, 141-151.
- Zheng, Z., Scott, S., Lukas, W., & Webb, M. (2000). A greedy algorithm for aligning DNA sequences. *J Comput Biol* , 203-14.
- Zhou, L., Meng, G., Zhu, L., Ma, L., & Chen, K. (2024). Insect antimicrobial peptides as guardians of immunity and beyond: a review. *International journal of molecular sciences*, 3835.
- Zhu, S., Peigneur, S., Gao, B., Umetsu, Y., Ohki, S., & Tytgat, J. (2014). Experimental conversion of a defensin into a neurotoxin: implications for origin of toxic function. *Molecular biology and evolution*, 546-559.
- Zhu, Y., Wang, W., Zheng, H., Chen, Y., Zhang, S., Sun, Y., & Wei, G. (2019). De novo transcriptome sequencing and analysis revealed the molecular basis of rapid fat accumulation by black soldier fly (*Hermetia illucens*, L.) for development of insectival biodiesel. *Biotechnology for Biofuels*, 194.