Layers of immunity: Deconstructing the *Drosophila* effector response

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Abstract

The host innate immune response relies on the cooperation of multiple defense modules. In insects and other arthropods, which have only innate immune mechanisms, four main immune-specific modules contribute to defense against microbial invaders: the Toll pathway, the Imd pathway, the melanization response, and phagocytosis by plasmatocytes. Our present understanding of their relative importance remains fragmented, as their contribution to host defense has never been simultaneously assessed across a large panel of pathogens. Here, we have taken advantage of newly-described immune mutants in a controlled genetic background to systematically delete these four immune modules individually, in pairs, or all four simultaneously. Surprisingly, flies simultaneously deficient in all four immune modules are viable, homozygous fertile, and display no overt morphological defects, suggesting these immune mechanisms are not strictly required for organismal development. With this new set of lines, we assessed the individual and collective contribution of each module to host defense against five viruses, three fungi, eight Gram-positive bacteria, and eight Gram-negative bacteria. Our findings show that these four modules largely function independently or additively in host defense, although synergistic effects can occur for select pairs of modules. Our study confirmed the importance of the Imd pathway against Gram-negative bacteria and the Toll pathway against Gram-positive bacteria and fungi, largely via the induction of effectors such as antimicrobial peptides (AMPs) and Bomanins (Bom), but also reveals an important role of melanization against viruses, and a contribution of phagocytosis against various germs. Additionally, by examining microbial load kinetics in different mutants, we provide insight into how these modules contribute to tolerance or resistance against specific microbes. Our study provides insights into the architecture of the *Drosophila* immune system, revealing differential requirements of immune modules according to each pathogen. The set of immune deficient lines provided here offers tools to better assess the role of these immune modules in host defense.

Keywords: Drosophila, innate immunity, effectors, phagocytosis, melanization, Imd, Toll

Introduction

The immune system is made up of a network of tissues and circulating cells whose primary function is to prevent and limit pathogenic infections (Danilova, 2006; Pradeu et al., 2024; Schmid-Hempel, 2021). Although immunity has been studied in many animal species, it has been extensively characterized in only a few, such as the nematode *Caenorhabditis elegans,* the fruit fly *Drosophila melanogaster*, zebrafish, rodents, and humans. In recent years, this traditional understanding of the immune system as a specialized group of cells and tissues has gradually changed to a more complex picture of highly redundant multilevel defense modules. The last few decades have revealed a great deal about the molecular mechanisms regulating each of these immune modules, from recognition and signaling to effector production (*e.g.* phagocytosis, antimicrobial peptides) (Pradeu et al., 2024). However, an immune response encompasses the concomitant activation of several modules and there has been little investigation of how modules contribute collectively to host defense. Immune research has traditionally focused on modules in isolation due to technical limitations. This article aims to fill this gap by analyzing the specific contributions of the main immune modules to the collective *Drosophila* systemic response.

The *Drosophila* systemic immune response consists of a set of humoral and cellular reactions in the hemolymph (insect blood) that occur when flies are systemically infected by bacteria, viruses, or fungi (Liegeois and Ferrandon, 2022; Westlake et al., 2024). A first facet of the systemic immune response is the production of host-defense effectors by the fat body and hemocytes that are secreted into the hemolymph. Among the best-characterized effectors are the antimicrobial peptides (AMPs), whose expression is rapidly induced to incredibly high levels upon infection in a race to control invading microbes (Hanson et al., 2019; Imler and Bulet, 2005). AMPs are small cationic peptides that exhibit antimicrobial activity and directly participate in microbial killing. Beyond AMPs, many other proteins and host defense peptides are secreted into the hemolymph, creating a hostile environment for invading pathogens through a variety of mechanisms. The systemic antimicrobial response is predominantly regulated at the transcriptional level by the Toll and Imd pathways (De Gregorio et al., 2002; Lemaitre et al., 1996). In *Drosophila*, the Toll pathway is activated by cell wall components (fungal glucans and Lysine-type peptidoglycan), as well as microbial proteases (Gottar et al., 2006; Issa et al., 2018; Leulier et al., 2003; Vaz et al., 2019). Toll signaling culminates in the activation of two NF-κB transcription factors, Dif and Dorsal, which regulate a large set of immune genes (Ip, 1993; Lemaitre et al., 1996). These encode small peptides such as the antifungal peptide Drosomycin, the Toll-regulated Bomanin peptides, and many other proteins (*e.g.* serine proteases, serpins, lipases) (Clemmons et al., 2015; De Gregorio et al., 2002; Fehlbaum et al., 1994; Ligoxygakis et al., 2003). Flies lacking the Toll pathway are viable but highly susceptible to infection by Gram-positive bacteria and fungi (Buchon et al., 2009; Lemaitre et al., 1996; Rutschmann et al., 2002). The Imd pathway is activated by DAP-type peptidoglycan produced by Gram-negative bacteria and a subset of Gram-positive bacteria (*e.g. Bacillus*) (Kaneko et al., 2004; Lemaitre et al., 1995; Leulier et al., 2003). The binding of peptidoglycan to receptors

of the Peptidoglycan Recognition Protein family (PGRP-SD, PGRP-LC, PGRP-LE) initiates an intracellular signaling cascade, which ultimately activates the NF-κB factor Relish (Choe et al., 2005; Iatsenko et al., 2016; Kaneko et al., 2006; Kaneko et al., 2006; Westlake et al., 2024). The Imd pathway regulates the expression of many genes, including those encoding antibacterial peptides and serine proteases (De Gregorio et al., 2002; Lemaitre et al., 1997). Imd deficient flies are viable but susceptible to Gram-negative bacterial infection.

In addition to humoral pathways, melanization and phagocytosis are two complementary mechanisms that contribute to host survival upon systemic infection. Melanization is an arthropod-specific immune response resulting in rapid deposition of the black pigment melanin at wound or infection sites and the concomitant production of microbicidal reactive oxygen species (Cerenius et al., 2008; Nappi et al., 2009; Westlake et al., 2024). The melanization and Toll pathways are co-activated at the level of the serine proteases Hayan and Persephone, which in turn activate Spatzle-processing enzyme (SPE) and serine proteases critical to cleavage of prophenoloxidases (PPOs) (Dudzic et al., 2019; Nakano et al., 2023; Shan et al., 2023). This is collectively referred to as the Toll-PO SP cascade (Westlake et al., 2024). The melanization reaction itself relies on the oxidation of phenols, resulting in the polymerization of melanin. Two PPOs (PPO1 and PPO2) are the primary catalysts for melanin synthesis during the melanization response to systemic infection. Flies deficient for both *PPO1* and *PPO2* are viable, but lack hemolymph phenoloxidase (PO) activity and exhibit susceptibility to certain Gram-positive bacteria and fungi (Binggeli et al., 2014; Dudzic et al., 2015).

Phagocytosis by both sessile and circulating plasmatocytes (*Drosophila* macrophage-equivalent cells) is thought to provide a complementary and important host defense (Melcarne et al., 2019; Ulvila et al., 2011). The use of hemocyte-deficient flies (via targeted expression of a pro-apoptotic gene in plasmatocytes) has shown that hemocytes contribute to survival upon systemic infection to certain bacterial species (Charroux and Royet, 2009; Defaye et al., 2009; Stephenson et al., 2022). Phagocytosis of bacteria greatly relies on two transmembrane receptors, NimC1 and Eater (Bretscher et al., 2015; Kocks et al., 2005; Kurucz et al., 2007; Melcarne et al., 2019). *NimC1, Eater* double mutant larvae are viable and their hemocytes can effectively encapsulate and melanize macroparasites; however, their hemocytes are non-sessile and nearly totally phagocytosis deficient. While *NimC1, Eater* larvae have elevated hemocyte numbers, adult flies have few hemocytes and fail to perform phagocytosis, providing a good tool to assess the role of cellular response (Melcarne et al., 2019; Melcarne, 2020).

We have scattered circumstantial evidence that each of these four immune modules are critical for defense against certain pathogens. However, we lack a global view of how, when, and to what extent each of these four immune modules contribute to host defense against specific pathogens, as they have never been simultaneously assessed. Moreover, the extent to which individual findings can be generalized to broad classes of pathogens remains unclear.

In this article, we have used a specific set of mutations principally affecting phagocytosis *(NimC1, Eater),* melanization *(PPO1, PPO2),* the Toll pathway *(spz),* and the Imd pathway *(Rel)* in a controlled genetic background to address their individual roles in host defense. We then recombined these and other mutations to generate double and even quadruple mutants of the four immune modules. This set of tools allowed us to systematically compare the survival of flies deficient in these responses against a broad panel of viruses, fungi, Gram-

positive bacteria, and Gram-negative bacteria, to reveal the relative contributions of each immune mechanism to resistance against these pathogens.

Materials and methods

Insect stocks

Precise details of *Drosophila* stocks used in this study are provided in **Table 1**. To minimize the influence of genetic background as a variable in this study, mutations were isogenized into the *DrosDel iso* w^{1118} genetic background (BDSC #5905) as described by Ferreira et al. (Ferreira et al., 2014; Ryder et al., 2004). Isogenized lines were generated and used for every mutant except the double mutant Toll and Imd *(*Rel^{E20}*,* spz^{rm7}*)* line, for which homozygous isogenized flies were not viable in our hands. Double mutants for $NimC1^{1}$*;* $Eater^{1}$ were poorly viable when isogenized in the *DrosDel iso* w^{1118} background, and so we used both isogenic and non-isogenic lines over the course of our study to facilitate investigation. Furthermore, $NimC1^{1}$*;* spz^{rm7}*,* $Eater^{1}$ triple mutants were homozygous lethal regardless of isogenization, and so could not be included. In addition to the immune module mutants, the Δ*AMP14* line bears a knockout of 14 different AMP genes, which includes the AMP families Drosomycin, Metchnikowin, Cecropin, Defensin, Drosocin, Attacin, and Diptericin, as described previously (Carboni et al., 2022; Hanson et al., 2019). The $Bom^{\Delta 55C}$ mutation was characterized in Clemmons et al. (Clemmons et al., 2015) and isogenic flies were generated in Hanson et al. (Hanson et al., 2019). For larval experiments, GFP-labelled CyO or TM3 balancers were used to enable selection of homozygous mutant larvae.

Microorganism cultures

Microbe strain information, microorganism classifications, and culture conditions are listed in **Table S1**. Bacterial cultures and *Candida albicans* were grown overnight shaking in appropriate media at 200rpm. *Aspergillus fumigatus* fungus was grown on Malt Agar at room temperature until sporulation. The entomopathogenic fungus *Beauveria bassiana* strain R444 was provided by Andermatt as spore preparations (BB-PROTEC) which were directly used in natural infections or dissolved in PBS for septic injury. Viruses DCV and FHV were kindly provided by Carla Saleh (Pasteur Institute, Paris), produced in S2 cells. The supernatant of S2 cells was titrated before being used for fly infections. Viruses SINV, DXV and IIV-6 were kindly provided by Ronald van Rij (Radboud University Medical Center, Nijmegen).

Heat-killed microbes were prepared by two repeats of boiling at 95°C for 30 minutes then freezing at -20°C for 30 minutes, before storage long term at -20°C. Microbe preparations were streaked onto agar plates to verify full efficiency of heat-killing before use in experiments.

Gene expression

Flies were inoculated by pricking at the junction of thoracic pleura with a needle dipped in a mixed pellet containing a 1:1 mixture of OD_{600} = 200 heat-killed *Escherichia coli* and *Micrococcus luteus* (final OD_{600} = 100 for each), and frozen at -20˚C 6h, 12h and 24h post-infection. Total RNA was then extracted from pooled samples of five flies using TRIzol reagent per manufacturer's protocol and resuspended in MilliQ dH2O. Reverse transcription was

173 performed using PrimeScript RT (Takara) with random hexamer and oligo dT primers.
174 Quantitative PCR was performed on a LightCycler 480 (Roche) in 96-well plates using Applied
175 Biosystems PowerUP SYBR green Master Mix (Applied Biosystems). Data points represent
176 pooled samples from three replicate experiments. Error bars represent one standard deviation
177 from the mean. Statistical analyses were performed using one-way ANOVA with Holms-Sidak
178 multiple test correction. Primers used in this study were:
179
180 Drs-F 5'- CGTGAGAACCTTTTCCAATATGAT-3'
181 Drs-R 5'- TCCCAGGACCACCAGCAT-3'
182 DptA-F: 5'-GCTGCGCAATCGCTTCTACT-3'
183 DptA-R: 5'-TGGTGGAGTGGGCTTCATG-3'
184 RPL32-F: 5'-GACGCTTCAAGGGACAGTATCTG-3'
185 RPL32-R: 5'-AAACGCGGTTCTGCATGAG-3'
186
187 **Ex vivo larval hemocyte phagocytosis assays**
188 Ex vivo phagocytosis assays were performed using a mix of equal volumes of *E. coli* and
189 *Staphylococcus aureus* AlexaFluorTM488 BioParticlesTM (Invitrogen), following
190 manufacturer's instructions; and see (Melcarne et al., 2019). Five L3 wandering larvae from
191 our newly generated mutant lines, or carrying the Hml-Gal4, UAS-GFP hemocyte marker as a
192 control, were bled into 150 uL of Schneider's insect medium (Sigma-Aldrich) containing 1 uM
193 phenylthiourea (PTU; Sigma-Aldrich). The hemocyte suspension was then transferred to 1.5
194 mL low binding tubes (Eppendorf, SigmaAldrich), and AlexaFluorTM488 bacteria
195 BioParticlesTM were added. The samples were vortexed, incubated at room temperature for 2
196 hours to allow phagocytosis and then placed on ice to stop the reaction. The fluorescence of
197 extracellular particles was quenched by adding 0.4% trypan blue (Sigma-Aldrich) diluted to 1/3
198 concentration. Phagocytosis was quantified using a flow cytometer (BD Accuri C6) to measure
199 the fraction of cells phagocytosing, and their fluorescence intensity. Isogenic wild-type iso w^{1118}
200 larvae and Hml-Gal4, UAS-GFP larvae with or without bacterial particles were used to define
201 the gates for hemocyte counting and the thresholds for phagocytosed particle emission. The
202 phagocytic index was calculated as follows:

$$\text{Fraction of haemocytes phagocytosing(f)} = \frac{[\text{number of haemocytes in fluorescence positive gate}]}{[\text{total number of haemocytes}]}$$

$$\text{Phagocyticindex}(PI) = [\text{Mean fluorescence intensity of haemocytes in fluorescence positive gate}] \times \text{f}$$

203
204 Finally, due to experimenter differences in total hemocytes collected and knock-on effects to
205 calculating phagocytic index, phagocytic index was normalized with the wild-type as a
206 reference set to 100% within experiment batch.
207
208 **Wounding experiment**
209 Clean injury was performed with a needle sterilized with an EtOH and PBS wash. For
210 imaging of the melanization reaction upon pricking, the thorax of 3-8 days old flies was
211 pricked using a sterile needle (diameter ~0.1mm). Pictures were taken 24h post-pricking and

212 categorized into normal, weak, or no melanization blackening seen at the injury site per
213 Dudzic et al. (Dudzic et al., 2019).
214
215 **Systemic infections and survival**
216 Systemic infections were performed by pricking 3- to 5-day-old adult males in the thorax with
217 a 0.1mm-thick needle dipped into a concentrated pellet of bacteria, yeast, or fungal spores. For
218 natural infections with *A. fumigatus,* flies were anesthetized and then shaken on a sporulating
219 plate of fungi for 30 seconds. For *B. bassiana*, flies were shaken for 30s in a tube containing an
220 excess of commercial spores sufficient to coat all flies uniformly, of which the flies later remove
221 the vast majority through grooming. At least two replicate survival experiments were performed
222 for each infection, though in specific cases some genotypes may have been included in only
223 one or no experiments. 20-35 flies were included per vial when possible, and maintained on
224 cornmeal fly media containing (per 1L): 6.2g agar, 58.8g cornmeal, 58.8g yeast, 60mL grape
225 fruit juice, 4.83mL propionic acid, and 26.5mL 100g/L moldex in pure ethanol. Survivals were
226 scored daily, and flies were flipped to new food vials 3 times per week. Due to challenges
227 working with genotypes of different viability, we could not include all genotypes in all
228 experimental replicates. Moreover, comparisons across genotypes required multiple levels of
229 consideration. Given the complexity of genotype-vs-reference-by-pathogen comparisons in our
230 study, needing to compare infections both to clean injury within genotype, and to wild-type
231 (WT, *DrosDel iso* w^{1118}) or *ΔITPM* (flies lacking all four immune modules) across treatments,
232 we chose to highlight survival differences only when they were: **i)** consistent across
233 experimental replicates; **ii)** of a consistent logic across comparable genotypes; for instance,
234 compound mutants containing *ΔMel* (*e.g. ΔIMD*, *ΔMel*) should be as or more susceptible to
235 infections as *ΔMel* alone if melanization is truly critical for defense; and **iii)** of a mean lifespan
236 difference ≥1.0 days after accounting for comparisons with unchallenged or clean-injury data.
237 Total experiments (N exp) and total flies per genotype are reported to provide summary
238 statistics per pathogen-by-genotype interaction. The mean lifespan reported in survival
239 summary data has a maximum of 7 or 10 days depending on experimental conditions. Kaplan-
240 Meier survival curves are provided in the supplementary information.
241
242 **Quantification of microbial load for growth kinetics**
243 3-8 days old flies were infected with the indicated microbe and concentration per OD_{600} as
244 described in **Table S1**, and allowed to recover. At the indicated time post-infection, flies were
245 anesthetized using CO_2 and surface sterilized by washing them in 70% ethanol. Ethanol was
246 removed, and then flies were homogenized using a Precellys bead beater at 6500 rpm for 30 s
247 in LB broth (*Providencia rettgeri*), BHI (*S. aureus*), or YPG (*C. albicans*) with 500 μL as pools
248 of 5 flies. These homogenates were serially diluted and 100uL was plated on LB, BHI or YPG
249 agar. Plates were incubated overnight, and colony-forming units (CFUs) were counted using a
250 Interscience Scan 500 plate scanning colony counter, and validated independently by manual
251 counts for a subset of plates to ensure accuracy. Statistical differences were tested using Brown-
252 Forsythe and Welch ANOVA tests with Dunnett's multiple test correction, which consider
253 unequal variance.
254

255 # Results

256 **A set of immune-deficient lines in the iso Drosdel background**

257 Mutations affecting only one of the four main modules of the *Drosophila* systemic immune
258 response have been described. These include *Relish*^{E20} (*Rel*^{E20}, deficient for the Imd pathway,
259 referred to here as Δ*IMD*), *spatzle*^{rm7} (*spz*^{rm7}, deficient for the Toll pathway, referred to here as
260 Δ*TOLL*), *PPO1*$^{\Delta}$, *PPO2*$^{\Delta}$ (no melanization, referred to here as Δ*Mel*), and *NimC1*^{1}; *eater*^{1}
261 (reduced phagocytosis, referred to here as Δ*Phag*). In addition to blocking phagocytosis, Δ*Phag*
262 flies have a defect in hemocyte sessility and a decreased number of hemocytes at the adult
263 stage (Bretscher et al., 2015; Melcarne et al., 2019; Melcarne, 2020). We have preferred the use
264 of *NimC1*^{1}; *eater*^{1} to study the contribution of hemocytes to defense rather than other
265 approaches with important indirect effects such as the pre-injection of latex beads to saturate
266 phagocytes (Elrod-Erickson et al., 2000), or expression of pro-apoptotic genes in plasmatocytes
267 (Charroux and Royet, 2009; Defaye et al., 2009).

268 All the mutations in this study were isogenized for at least seven generations into the
269 DrosDel *iso w*^{1118} genetic background (Ferreira et al., 2014; Ryder et al., 2004). We then
270 generated by recombination six of the seven possible combinations of flies simultaneously
271 lacking two immune modules, with the exception of *ΔTOLL*, *ΔPhag* as *NimC1*^{1}; *eater*^{1}, *spz*^{rm7}
272 flies were not viable (see fly strains in **Table 1**). Of note, in our hands recombinant *Rel*^{E20},
273 *spz*^{rm7} flies were not viable in the DrosDel isogenic background, necessitating use of non-iso
274 flies to assess host defense in *ΔIMD*, *ΔTOLL* flies. Over the course of our study, isogenic
275 *NimC1*^{1};*eater*^{1} flies also displayed viability issues, and specific experiments used non-isogenic
276 flies to improve confidence in phenotypes; these experiments are noted in figure captions. We
277 used two approaches to simultaneously delete the Toll and melanization modules: **i)** *PPO1*$^{\Delta}$,
278 *PPO2*$^{\Delta}$; *spz*^{rm7} flies (referred to as *ΔTOLL*, *ΔMel*^{PPS}) and **ii)** flies carrying a small viable deletion
279 *Hayan-psh*^{Def} (referred to as *ΔTOLL*, *ΔMel*^{HP}) which removes two clustered serine protease
280 genes, *Hayan,* and *persephone,* blocking the TOLL-PO SP cascade that regulates both humoral-
281 Toll and melanization (Dudzic et al., 2019; Westlake et al., 2024). This effectively deletes the
282 Toll and melanization pathways using only one deletion event on the *X* chromosome.
283 Excitingly, we succeeded in generating a fly line deficient for all four immune modules with
284 the genotype: *Hayan-psh*^{Def}; *NimC1*^{1}; *eater*^{1}, *Rel*^{E20} hereafter named *ΔITPM (ΔIMD, ΔTOLL,*
285 *ΔPhag, ΔMel).* These flies were homozygous viable (very poor viability), and fertile, despite
286 major deficiencies in these four important systemic immune mechanisms. These flies allow us
287 to monitor survival kinetics and pathogen growth in the near-total absence of an immune
288 system. Finally, we added two iso fly lines in our analysis that delete key host defense peptides
289 downstream of the Toll and Imd pathways: **i)** *Bom*$^{\Delta 55C}$, a short deletion removing ten Bomanins
290 at position 55C, which causes a major susceptibility to infection by Gram-positive bacteria and
291 fungi (Clemmons et al., 2015), and **ii)** Δ*AMP14*, a compound fly line of eight mutations
292 removing 14 AMP genes (4 Cecropins, 4 Attacins, 2 Diptericins, Drosocin, Defensin,
293 Drosomycin, and Metchnikowin), which causes a major susceptibility to infection by Gram-
294 negative bacteria (Carboni et al., 2022; Hanson et al., 2019). This set of immune-deficient fly
295 lines in a defined genetic background provides a unique tool kit to systematically address the
296 individual and collective contributions of immune modules to defense against a variety of
297 pathogens.
298

299 **Imd, Toll, Phagocytosis, and Melanization modules function largely independently**
300 The Imd, Toll, Phagocytosis, and Melanization modules are interconnected (Westlake et al.,
301 2024). For instance, the Toll and Imd pathways both regulate a subset of genes involved in
302 melanization (De Gregorio et al., 2002; Ligoxygakis et al., 2002). Moreover, one module can
303 indirectly affect another. For instance, a lack of melanization could increase the bacterial load
304 after infection, resulting in higher activation of the Toll and Imd pathways (Binggeli et al.,
305 2014). In the first step, we analyzed the activation of each of the four modules in single- and
306 multiple-module deficient flies using classical readouts of these modules. This also allowed
307 monitoring of the possible effects of deleting one module on the activity of another. We
308 analyzed the immune response in single module deficient flies by monitoring **i)** the expression
309 of the antibacterial peptide gene *Diptericin* (*DptA* - a target of the Imd pathway), and **ii)** the
310 antifungal peptide gene *Drosomycin* (*Drs* - a target of the Toll pathway) upon systemic
311 infection with a mixture of heat-killed *Escherichia coli* and *Micrococcus luteus*, **iii)** ex vivo
312 phagocytosis of *E. coli* and *Staphylococcus aureus* coated bioparticles incubated with larval
313 hemocytes, and **iv)** melanization at the injury site of adult flies. This analysis first confirmed
314 that Δ*IMD*, Δ*TOLL*, Δ*Phag,* and Δ*Mel* flies are deficient for Imd, Toll, phagocytosis, and
315 melanization respectively (**Figure 1A-D**). We note that the expression level of *DptA* was
316 roughly similar to the wild-type in Δ*TOLL* and Δ*Mel* flies, however, *ΔPhag* flies show reduced
317 *DptA* induction at 6h compared to WT (~50%) (**Figure 1A**). This represents an immense
318 induction of *DptA* after infection, but may suggest either a lag or lesser overall Imd response in
319 *ΔPhag* flies. *DptA* expression in *ΔPhag* was, however, comparable to WT at 12h and 24h. On
320 the other hand, *Drs* induction at 6h in *ΔMel* flies was significantly higher than WT (~1.6x), as
321 found previously using live *M. luteus* infection (Binggeli et al., 2014) (**Figure 1B**). The
322 phagocytic assay confirmed that the Δ*Phag* mutant was significantly impaired in its ability to
323 phagocytose bacterial bioparticles. Furthermore, Δ*IMD,* and Δ*Mel* mutant larvae had a
324 phagocytic index similar to WT (**Figure 1C**), while Δ*TOLL* had a higher phagocytic index, but
325 with high variance. As expected, there was no apparent melanization at the site of the injury in
326 Δ*Mel* flies while blackening was readily observed in the wild-type and in other single-module
327 mutants (**Figure 1D**). Collectively, this analysis indicates that each of the four immune modules
328 can be selectively blocked in the isogenic DrosDel background without interfering with the
329 competence of the others. We did note in some cases a modest change in module activity in the
330 absence of another (higher Toll activity in Δ*Mel,* lower IMD activity in Δ*Phag,* and higher
331 phagocytic activity in Δ*TOLL*), findings that require further exploration to establish
332 consistency.
333 We then repeated our analysis using our set of module compound mutants.
334 Unsurprisingly, all compound flies displayed the expected immune deficiencies, validating the
335 lines we constructed. This analysis confirms that *ΔITPM* flies, although viable, are indeed fully
336 immune deficient for the four host defense modules. We did not detect any striking synergistic
337 or antagonistic effects where deleting two modules had a vastly different impact compared to
338 the additive effect of deleting the modules individually. Notably, a small level of *Drs* expression
339 is retained in Δ*TOLL* mutant flies, but was fully abolished in Δ*IMD,* Δ*TOLL* flies (**Figure 1A**
340 **vs 1E**), confirming observations that the *Drs* gene receives a small input from the Imd pathway
341 (Leulier et al., 2000). The Δ*TOLL,* ΔMel^{PPS} and, Δ*TOLL,* ΔMel^{HP} flies display *Drs* induction at
342 6h that is resolved to unchallenged WT levels by 12h, an expression pattern consistent with

343 mediation of this induction by early input from the IMD pathway in these backgrounds. Taken
344 together, all the compound mutant flies behave largely as expected given the sum effects of
345 their individual mutations, confirming that these modules largely function independently.
346
347 **Both the Melanization and TOLL modules contribute to lifespan and survival to**
348 **wounding**
349 Before assessing resistance to infection, we monitored fly survival at 25°C and 29°C to
350 determine if module mutations impact viability in the absence of challenge. At 25°C, no single
351 module mutants died significantly more than wild-type in the absence of challenge within the
352 ten-day window (**Figure 2A**). However, even in the absence of challenge all compound mutants
353 other than Δ*IMD, ΔTOLL* suffered at least 25% cumulative mortality by 10 days post-eclosion,
354 while Δ*TOLL, ΔMel*^{PPS} and Δ*ITPM* flies suffered even greater cumulative mortality. Similar
355 trends were observed at 29°C with slightly elevated mortality (**Figure 2supp1**). The
356 observation that *ΔTOLL, ΔMel*^{PPS} (*PPO1*^{1}*,PPO2*^{1}*, spz*^{rm7}) flies have shorter lifespan compared
357 to *ΔTOLL*, *ΔMel*^{HP} (*Hayan-psh*^{Def}) could be explained by residual Toll and/or PO activity in
358 *Hayan-psh*^{Def} or additional indirect effects in *ΔTOLL, ΔMel*^{PPS} flies. As such, the combined
359 effect of Toll and Melanization deficiencies in unchallenged and clean injury survival is best
360 assessed by considering signals from both genotypes. *ΔITPM* flies (*Hayan-psh*^{Def}*; NimC1*^{1}*;*
361 *Eater*^{1}*, Rel*^{E20}) have the lowest unchallenged survival rate of genotypes included in this study.
362 Taken together, the Imd, Toll, Melanization, and Phagocytosis modules contribute little to
363 lifespan maintenance individually, but are crucial to maintain lifespan collectively (**Figure 2B**).
364 Systemic infections were performed by septic injury (pricking with a contaminated
365 needle). We therefore monitored the survival of single and double mutant flies after a clean
366 injury to disentangle the effects of injury from those of infection. We found a cumulative
367 mortality of ~50% for Δ*Mel* flies by day 10 (**Figure 2C**), while other single module mutants
368 displayed relatively little mortality over the 10-day window; *ΔIMD* had notable late-onset
369 mortality in a minority of experimental replicates, which may be due to stochastic dysbiosis
370 effects during aging (Hanson and Lemaitre, 2023; Marra et al., 2021). Double mutant *ΔIMD,*
371 *ΔTOLL* and Δ*IMD, ΔPhag* flies retained a high survival rate. Consistent with an importance of
372 melanization in the injury response (Binggeli et al., 2014), all the compound mutants affected
373 in melanization were susceptible to clean injury, with similar mortality seen in *ΔPhag, ΔMel*,
374 *ΔIMD, ΔMel*, *ΔTOLL, ΔMel*^{PPS}, *ΔTOLL, ΔMel*^{HP}, and *ΔITPM* flies. Thus, while these genotypes
375 differ in their base lifespan, their viability upon injury is remarkably similar. These results
376 emphasize the interconnectedness of the melanization response with every other immune
377 module for maintaining health after injury.
378 Similar results were obtained for both unchallenged and clean injury lifespans at 29°C.
379 Of note, *ΔTOLL*, *ΔMel*^{PPS} suffered significantly greater mortality in unchallenged conditions
380 than other genotypes, even including *ΔITPM* flies (**Figure 2supp1**). Other compound mutant
381 genotypes displayed a continuum of mortality in unchallenged conditions between *ΔITPM*
382 (~50% cumulative mortality) and *ΔTOLL, ΔMel*^{HP} (~15% cumulative mortality). Upon clean
383 injury, results were broadly consistent with 25°C trends, with a more uniform mortality across
384 all genotypes.

385 Collectively, our study highlights that all these immune modules contribute somewhat
386 to the survival of flies upon clean injury, with a major role of the melanization response in
387 maintaining lifespan and survival to injury. In the next steps of our study, we compared survival
388 upon infection to both unchallenged and clean injured flies to distinguish the impact of
389 microbial infection from the injury itself.
390
391 **Multiple mechanisms contribute to resistance to microbial infection**
392 To compare the contribution of each module to host defense, we performed infection
393 experiments of wild-type and single- and multiple-module flies following septic infection with
394 five viruses, eight Gram-positive bacteria, eight Gram-negative bacteria, septic injury for two
395 fungal species, and natural infection (*i.e.* spore deposition on the cuticle) for two fungal species.
396 Temperature, dose, and days monitored are shown in **Figure 3**, and see **Table S1** for other key
397 parameters. Survival curves are shown in **Supplementary file 1** for each pathogen and results
398 of these survivals are summarized in **Figure 3**.
399 We performed infection experiments with 24 viruses and microbial species: DCV, FHV,
400 DXV, IIV-6, SINV, *Aspergillus fumigatus*, *Beauveria bassiana*, *Candida albicans*,
401 *Mycobacterium marinum*, *Corynebacterium diphtheriae, Micrococcus luteus, Streptococcus*
402 *pneumoniae, Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes, Bacillus*
403 *subtilis, Vibrio parahemolyticus, Providencia rettgeri, Providencia burhodogranariea,*
404 *Pectobacterium carotovorum Ecc15, Klebsiella pneumoniae, Enterobacter cloacae,*
405 *Escherichia coli, and Salmonella enterica ser. typhimurium.* Collectively, this represents
406 infections with 24 pathogens across 14 genotypes and two infection routes (~350 genotype-by-
407 pathogen interactions). When combined with comparisons to clean injury and unchallenged
408 controls to validate if a lifespan difference is meaningful, and to wild-type or *ΔITPM* treatments,
409 this inflates to 1000+ interactions. We therefore focused our survival analysis on major effects.
410 We report the core summary statistics and overt trends, and comment only where difference in
411 mean lifespan between treatments and reference genotypes or controls was a minimum of 1
412 day.
413 Our study confirms that the Imd pathway plays a major role against all tested Gram-
414 negative bacteria, as Δ*IMD* flies succumb more rapidly than wild-type flies to all of them. This
415 pathway also contributes, albeit to a lesser extent, to survival against the Gram-positive bacteria
416 *B. subtilis* and *L. monocytogenes,* which bear DAP-type peptidoglycan at their membrane that
417 triggers Imd activation (Kaneko et al., 2004; Leulier et al., 2003). We were interested to see
418 the impact of our Δ*IMD* flies in host defense to viruses as the specific mutation we used (Rel^{E20})
419 deletes a transcription factor common to both the canonical Imd pathway and the recently-
420 appreciated cGLR-Sting-Relish pathway with roles in viral defense (Goto et al., 2018). We
421 found that Δ*IMD* mutant flies were somewhat susceptible to the four viruses DCV, FHV, DXV,
422 and IIV6, but not SINV, agreeing with previous studies showing a reduction in lifespan of 1-2
423 days for *Rel* mutant flies after viral infection (Costa et al., 2009; Goto et al., 2018; Sansone et
424 al., 2015).
425 This study also confirms a prominent role of the Toll pathway in survival upon infection
426 by all virulent Gram-positive bacteria and fungi upon septic injury. Less virulent microbes and
427 infectious routes (such as *A. fumigatus* by natural infection) did not result in increased mortality
428 in individual *ΔTOLL* mutant flies. A contribution of the Toll pathway to survival was also

429 observed for some Gram-negative bacteria, including a minor contribution to survival upon *P.*
430 *rettgeri* infection, and a major contribution after *V. parahemolyticus* infection. In addition, we
431 found a susceptibility of *ΔTOLL* flies to FHV, DXV, and IIV6, suggesting Toll-regulated
432 effectors mediate defense against some viral infections.
433 Use of *ΔPhag* mutants reveals a role of the cellular response in survival against the
434 fungus *B. bassiana*. *ΔPhag* flies further show a minor susceptibility to *S. aureus,* and a subset
435 of Gram-negative bacteria (*P. rettgeri*, *Ecc15,* and *E. coli*). Susceptibilities to Gram-negative
436 bacteria could result from the observed lag in Imd pathway activation (Figure 1A); indeed,
437 previous studies have shown a full *DptA* response is pivotal for defense against *P. rettgeri*
438 (Hanson et al., 2023, 2019; Unckless et al., 2016), and lifespans are greatly impacted by subtle
439 difference for pathogens with intermediate levels of virulence (Duneau et al., 2017). A role for
440 Imd in explaining *ΔPhag* susceptibilities does not detract from the importance of the cellular
441 response, but instead offers a route to explore mechanism for these phenotypes.
442 Surprisingly, our study reveals a relatively consistent role of melanization against all the tested
443 viruses, notably so for DXV, IIV-6, and SINV. Δ*Mel* flies also display a minor increased
444 susceptibility to *B. bassiana* natural infection and septic injury. As previously described
445 (Dudzic et al., 2019), *ΔMel* flies are highly susceptible to the Gram-positive bacterium *S.*
446 *aureus*, rivaling the susceptibility of *ΔITPM* flies. *ΔMel* flies further show a strong
447 susceptibility to *P. rettgeri* rivalling *ΔIMD* flies, and also a minor susceptibility to infection by
448 other Gram-negative bacteria (*P. burhodogranariea, E. coli*).
449 Collectively, the use of single module mutants confirms the major role of the Imd
450 pathway against Gram-negative bacteria and Toll against virulent Gram-positive bacteria and
451 fungi. Interestingly, melanization was consistently important to survive viral infection, and
452 plays a prominent and important role in defense against specific bacterial species. While it was
453 almost never the most critical module, phagocytosis and the cellular response contributes to
454 survival against various germs. Use of single module deficient flies alongside *ΔITPM* flies
455 reveals that survival to some of the microbes tested relies almost entirely on one pathway (Imd
456 – *Ecc15, K. pneumoniae, E. cloacae, P. burhodogranariea*; Toll – *E. faecalis*), or can receive
457 notable contributions from two pathways (Toll and IMD – *L. monocytogenes, B. subtilis, V.*
458 *parahemolyticus*; Toll and Phagocytosis – *A. fumigatus* and *B. bassiana* natural infection*;* Imd
459 and Melanization - DCV). Strikingly, many pathways contribute to host defense to *S. aureus*
460 and *P. rettgeri*.
461
462 **Contribution of AMP and Bomanins to Toll and Imd activities**
463 Recent studies have described a prime importance of antimicrobial peptides (AMPs) and host
464 defense peptides (HDPs) in responding to infection (Clemmons et al., 2015; Hanson, 2024;
465 Hanson et al., 2019). These peptides are the principal effectors of the Toll and Imd pathways,
466 yet mutations for AMPs and HDPs typically have not been tested alongside mutations for both
467 the Imd and the Toll pathways across pathogens, as studies have typically included just the
468 single relevant pathway mutation as a positive control. Thus, the importance of AMPs and
469 HDPs relative to the pathways that regulate them has been only partially investigated. We used
470 *ΔAMP14* and *ΔBom* flies to assess the extent to which AMPs and Bomanins mediate Toll and
471 Imd contributions to defense (**Figure 3**). Here we found that AMPs are critical for survival to

472 infection against all Gram-negative bacteria tested, often rivaling the susceptibility of *ΔIMD*
473 flies. Notably, *ΔAMP14* flies were also somewhat susceptible to the yeast *C. albicans,* and the
474 Gram-positive bacteria *B. subtilis* and *M. luteus*, reinforcing minor susceptibilities seen
475 previously (Carboni et al., 2022; Hanson et al., 2019). Interestingly, we found that *ΔAMP14*
476 survivals to DCV, FHV, DXV, and IIV6, largely paralleled those of *ΔIMD* flies (Figure 3,
477 Supplemental File 1), suggesting Imd-mediated survival phenotypes rely greatly on the
478 presence of the seven classical AMP families.
479 On the other hand, *ΔBom* flies were susceptible to septic injury by *B. bassiana* and also
480 somewhat to *C. albicans,* consistent with previous studies (**Supplementary file 1**), as well as
481 all virulent Gram-positive bacteria, consistent with their important role downstream of the Toll
482 pathway (Clemmons et al., 2015; Lindsay et al., 2018; Xu et al., 2023). In addition, *ΔBom* flies
483 paralleled the *ΔTOLL* susceptibility to DCV, FHV, and DXV, but were not susceptible to IIV6
484 like *ΔTOLL*.
485 Collectively, our study confirms the key role of antimicrobial peptides downstream of
486 the Imd pathway in the defense against Gram-negative bacteria and a primary role of Bomanins
487 downstream of Toll in defense against fungi and Gram-positive bacteria, with little input to
488 survival against Gram-negative bacteria. We additionally find these secreted peptides can
489 contribute to survival after certain viral infections.
490
491 **Immune modules mostly contribute additively to host defense**
492 The results described above reveal the key contribution of single modules and immune
493 effectors to host defense. However, they did not assess possible additive, synergistic, or
494 antagonistic contribution of these four modules. Moreover, they did not monitor to what
495 extent these modules are important in the absence of other modules. To address this, we
496 compared the survival of single-module deficient flies to flies lacking two modules, and
497 *ΔITPM* flies lacking all four modules (**Figure 3**).
498 We first observed that *ΔITPM* flies, which lack the four modules, were always as or
499 more susceptible than single- and double-module deficient flies. In multiple microbe-specific
500 cases, single or double mutants rivalled the susceptibility of *ΔITPM* flies. In particular, the
501 susceptibility of *ΔIMD*-flies to most Gram-negative bacteria rivalled *ΔITPM,* and for some
502 germs *ΔTOLL* (*L. monocytogenes*, *E. faecalis*, *B. bassiana* injury) or *ΔMel* (*S. aureus*) alone
503 was comparable to *ΔITPM*. In other cases, however, combined loss of two pathways was
504 necessary to rival *ΔITPM* susceptibilities. For instance, *ΔIMD, ΔTOLL* deletion causes
505 complete *ΔITPM*-like susceptibility to *B. subtilis*, *M. luteus*, and *E. coli*, and near-complete
506 susceptibility to *S. typhimurium*. *ΔIMD, ΔTOLL* further causes increased susceptibility to
507 most viruses compared to its single module counterparts. In these cases, there is clear synergy
508 or additivity of the contributions of the two modules, as either pathway alone results in only
509 minor or no susceptibility. We also observed intriguing differences between our two versions
510 of *ΔTOLL, ΔMel* flies, as *ΔTOLL,* ΔMel^{PPS} tended to succumb to infection to a greater extent
511 than *ΔTOLL,* ΔMel^{HP}, consistent with the differences observed upon clean injury (**Figure 2**).
512 The *ΔTOLL*, ΔMel^{PPS} displayed a minor increase in susceptibility compared to *ΔTOLL*,
513 ΔMel^{HP} to *A. fumigatus* natural infection, and more prominent susceptibilities to the yeast *C.*

514 *albicans*, the Gram-positive bacteria *M. marinum*, *M. luteus* and also to Gram-negative
515 bacteria *K. pneumoniae*, *E. coli*, *P. rettgeri* and *P. burhodogranariea*.
516 Collectively, use of double-module deficient flies uncovers contributions of immune
517 modules to host defense that were masked by other modules. While *ΔIMD* and *ΔTOLL*
518 deficiencies sometimes displayed a synergistic effect (*i.e.* little mortality of single mutants,
519 but *ΔITPM*-like mortality of double mutants), for combinations of other pathways the results
520 were more often minor with a cumulative effect on susceptibility, suggesting most modules
521 contribute to defense independently. The use of two variations of *ΔTOLL*, *ΔMel*, with *ΔTOLL*,
522 ΔMel^{PPS} having a stronger impact than *ΔTOLL*, ΔMel^{HP}, also highlights how the level of
523 pathway disruption can affect infection outcomes, particularly for certain fungal or bacterial
524 pathogens like *Providencia* species that may cleave host immune proteases (suggested by
525 Duneau et al., 2017; Issa et al., 2018).
526
527 **Timing of Toll, Imd, Phagocytosis, and Melanization contribution to host defense**
528 Host defense programs can rely on resistance mechanisms that directly target or limit growth
529 of pathogens (Howick and Lazzaro, 2017; Lafont et al., 2021; Medzhitov et al., 2012). When
530 effectors reach a critical concentration threshold that promotes resistance, pathogen growth is
531 inhibited; for instance, the time to pathogen control of *P. rettgeri* is ~7 hours in *Drosophila*,
532 relying heavily on the expression and production of DptA (Duneau et al., 2017; Hanson et al.,
533 2023). Phagocytosis and the melanization response are often described as providing near-
534 immediate immune protection (Haine et al., 2008), with reactions ex vivo progressing within
535 minutes. Meanwhile activation of the Imd and Toll pathways takes hours to reach peak
536 concentrations of host defense peptides (Uttenweiler-Joseph et al., 1998). Thus, we were
537 curious if different mutations would alter time of action to control microbial growth. We
538 therefore measured the microbial growth rate for *P. rettgeri, S. aureus,* and *C. albicans* at
539 different time points post-infection in wild-type and single module mutant flies as these
540 pathogens were combatted by several immune modules in our survival data.
541 Agreeing with contributions to survival, flies singly deficient for IMD, Phagocytosis,
542 or Melanization modules display a higher bacterial load than wild-type when infected with *P.*
543 *rettgeri* **(Figure 4A)**, which was apparent at 12 hours post-infection. Our observation that
544 *ΔPhag* showed impaired IMD signaling at 6h (**Figure 1A**) may explain the contribution of
545 phagocytosis to *P. rettgeri* microbial growth control with the same timing as *ΔIMD* flies. We
546 observed that *ΔIMD* flies, *ΔMel* flies, and *ΔITPM* flies, each of which succumb completely to
547 *P. rettgeri* infection, also show high and consistent microbe loads at 24h that are indicative of
548 sepsis-induced death. On the other hand, wild-type, *ΔTOLL*, and *ΔPhag* flies show a greater
549 stochasticity in bacterial load at 24h that is consistent with a fraction of individuals surviving
550 the infection by controlling the pathogen. This indicates deletion of the Toll pathway and
551 phagocytosis does not fully ablate resistance mechanisms essential for combatting *P. rettgeri*.
552 In the case of *S. aureus* (**Figure 4B**), we observed some individuals with higher bacterial
553 loads in Δ*Phag*, and Δ*IMD* flies at 2h and 6h, which was not seen in wild-type, Δ*TOLL,* or Δ*Mel*
554 flies. This suggests that the Imd pathway can suppress early *S. aureus* growth, and possibly also
555 phagocytosis, although this difference in Δ*Phag* could ultimately stem from reduced IMD
556 induction. Interestingly, flies lacking melanization displayed the highest susceptibility, but did

557 not depart from wild-type or other modules in pathogen load until 12h post-infection. While
558 the melanization reaction is a rapid response, these results suggest that the killing activity of
559 the melanization response acts with slower kinetics. In *ΔITPM* flies, we observed earlier *S.*
560 *aureus* growth at 2h and 6h as in *ΔIMD* and *ΔPhag* flies. However, *S. aureus* loads at 12-24h
561 were more comparable in *ΔITPM* flies to *ΔMel* flies. These trends agree both with survival data
562 kinetics, and an independent action of each of these pathways in their resistance effects against
563 *S. aureus*. This suggests that *ΔITPM* flies succumb even more quickly than individual module
564 mutants due to loss of multiple resistance mechanisms with different kinetics of activation that
565 independently contribute to defense.
566 For *C. albicans* (**Figure 4C**), mortality begins 2-3 days post-infection in Δ*TOLL*
567 mutants, but takes place at later time points for Δ*IMD,* Δ*Phag,* and *ΔMel* flies. Microbial growth
568 kinetics show high *C. albicans* loads at 24h in Δ*TOLL,* consistent with a previous study (Hanson
569 et al., 2019) and observations done with *Candida glabrata* (Quintin et al., 2013). Interestingly,
570 a few Δ*IMD* flies also showed elevated growth of *C. albicans* at early time points. This suggests
571 Imd-regulated effectors may help to suppress initial *C. albicans* growth, while Toll-regulated
572 genes (*e.g.* Bomanins, Drs) contribute to this pathogen suppression to a greater extent at later
573 time points. On the other hand, Δ*Phag* and Δ*Mel* flies did not show any increased *C. albicans*
574 load within the 24h time window, despite onset of mortality at later time points. Finally, Δ*ITPM*
575 flies displayed more consistent and rapid growth of *C. albicans* than *ΔTOLL* or *ΔIMD* alone,
576 particularly visible at 12-24h post-infection, consistent with independent contributions of both
577 Toll and Imd in resistance to this yeast.
578 Collectively, we observed that microbial growth in each module mutant parallels
579 susceptibility, indicating that they contribute to resistance. Surprisingly we did not observe an
580 early role in pathogen killing for melanization despite the rapid blackening response mediated
581 by melanization in ex vivo assays (Nakhleh et al., 2017). We further recover stochasticity in
582 microbe loads across genotypes, as expected when only a subset of individuals suppress the
583 pathogen and survive the infection, resulting in bimodal outcomes (Duneau et al., 2017). The
584 more rapid and consistent microbial growth kinetics of *ΔITPM* flies, particularly visible at 18h,
585 further demonstrate that the modules contribute to resistance collectively.
586
587 **Contributions of individual modules to disease tolerance**
588 A metric increasingly used to delineate roles of resistance and tolerance is the Pathogen Load
589 Upon Death (PLUD) (Duneau et al., 2017; Duneau et al., 2025). PLUD is affected by both the
590 virulence of the pathogen and tolerance of the host to survive to a given pathogen burden before
591 succumbing to infection. We monitored PLUD for the same pathogens used to determine the
592 timing of defense modules: *P. rettgeri, S. aureus,* and *C. albicans.* The PLUD of both *P. rettgeri*
593 and *S. aureus* was overall similar in wild-type and single-module mutant flies **(Figure 5A-B)**.
594 Notably, there was a greater stochasticity in *P. rettgeri* infections trending towards lower PLUD
595 in Δ*ITPM* flies, suggesting a stochastic reduction in disease tolerance of *ΔITPM* flies to this
596 bacterium, although this was not significant compared to wild-type ($P > .05$).
597 For *C. albicans*, the distribution of PLUD values for *ΔPhag* flies was markedly different
598 from wild-type, also seen to some extent for *ΔMel*. Interestingly, flies with these genotypes
599 suppress *C. albicans* growth well (**Figure 4C**). Yet here we recovered many individuals from

both of these module-deficient lines that died with a far lower PLUD (**Figure 5C**). Taken together, this suggests that the humoral immune pathways, particularly Toll, contribute to resistance against *C. albicans*. However, the cellular response and melanization reaction instead regulate tolerance to *C. albicans* infection. The use of Δ*ITPM* flies further emphasizes that when both resistance (Toll, Imd) and tolerance (Phagocytosis, Melanization) mechanisms are deficient, the loss of resistance plays a primary importance, and can mask observations of reduced tolerance.

Collectively, monitoring of PLUD suggests that some modules, specifically phagocytosis and melanization, contribute to tolerance of certain infections. Importantly, this tolerance effect was only seen in genotypes that are able to resist infection. These findings demonstrate the collective contribution to resistance and/or tolerance of the four immune modules studied here.

Discussion

In this article, we have generated a set of single and compound immune module deficient fly lines in a controlled genetic background. Using various assays, we confirmed the validity of our lines, revealing that each module can be activated independently. We did, however, observe higher Toll activation in Δ*Mel* flies upon systemic infection with dead bacteria. Future studies may reveal that this higher Toll activity in Δ*Mel* flies is due to interaction with a regulatory pathway, or reflects higher persistence of microbial elicitors (*e.g.* peptidoglycan) that activate the Toll pathway in melanization-deficient flies. We also observed reduced Imd activation in Δ*Phag* flies at 6 hours post-infection, which could reflect a role for hemocytes in stimulation of the Imd systemic response. In this study, we used $NimC1^{1}$; $eater^{1}$ double mutants to assess the cellular response. These flies have defects in phagocytosis, but also hemocyte adhesion and sessility (Melcarne et al., 2019). $NimC1^{1}$; $eater^{1}$ larvae also have increased hemocyte number at the larval stage, but Hml positive cells are rapidly lost in adults (Melcarne, 2020). Despite these limitations, we believe that the mutations we have used here are among the best available to assess the roles of these four modules to host defense. Surprisingly, we were able to produce a fly line lacking all four main defense mechanisms of the systemic immune response, indicating that none of these modules is essential for survival. Theoretically, Δ*ITPM* flies are almost completely immune deficient, but they are still able to clot wounds, activate the JNK and JAK-STAT pathways that mediate the wound healing response, and retain constitutive immune defense molecules that could provide a certain degree of protection. Consistent with several studies (Capilla et al., 2017; Carvalho et al., 2014; Rämet et al., 2002), we show that Toll and melanization contribute synergistically to wound healing in adults. However, the observation that Δ*Phag,* Δ*Mel* or Δ*IMD,* Δ*Mel* double mutant flies are also more susceptible to clean injury than Δ*Mel* flies indicates that the Imd pathway and phagocytosis also contribute to wound healing. Thus, although immune deficient fly lines for the four modules are viable, the use of compound module mutants and Δ*ITPM* flies reveals a clear role of the immune system in response to wounding and lifespan maintenance.

Use of single- and double module-deficient flies provides key insights on the mechanisms used by *Drosophila* to combat infection. We confirm previous studies revealing the role of Imd against Gram-negative bacteria, Toll against Gram positive bacteria and virulent fungi, and an importance of Melanization and Phagocytosis against specific pathogens

644 (Charroux and Royet, 2009; Defaye et al., 2009; Garg and Wu, 2014; Nehme et al., 2011).
645 Surprisingly, the Melanization module was consistently important in survival to viral infection.
646 It seems unlikely that prophenoloxidase activity in the hemolymph can combat intracellular
647 viral agents. We instead speculate this contribution of melanization to viral defense could be
648 due to its role in wound healing, or perhaps autotoxic contributions of melanization reaction
649 intermediates that fail to convert in phenoloxidase-deficient flies. We additionally recovered a
650 role of Imd signaling (i.e. *Relish*) in antiviral defense, which was expected given recent
651 characterizations of cGLR-Sting-Relish antiviral immunity (Ai et al., 2024; Cai et al., 2022;
652 Goto et al., 2018). However, susceptibilities of Rel^{E20} flies were paralleled by Δ*AMP14* in all
653 cases, suggesting AMPs largely mediate this susceptibility. While Sting regulates a number of
654 genes likely important for antiviral defense (Goto et al., 2018), the susceptibility we observe
655 here could be a direct action of AMPs on enveloped viruses as described in some studies (Feng
656 et al., 2020; Huang et al., 2013; Yasin et al., 2004), or an indirect effect, such as a need to
657 regulate gut microbes after the damage induced by viral replication (Marra et al., 2021). We
658 additionally used dual modes of infection for the fungus *B. bassiana,* finding that both
659 Melanization and Toll modules have a role in both infection modes. However, our study reveals
660 that Bomanin effectors explain most of the Toll contribution upon septic injury but not natural
661 infection by *B. bassiana*. This observation is in line with previous observations showing
662 differential importance of effectors or modules according to infection route (Martins et al.,
663 2013).
664 Our double module mutant analysis reveals that most modules contribute additively to
665 host defense. This is consistent with largely independent function of these modules. However,
666 we noted some instances of synergy between two pathways, notably Toll and Imd. Synergy
667 between Toll and Imd can be explained by the fact that many immune-inducible regulated genes
668 receive input of both the Imd or Toll pathways; some like *Metchnikowin*, *Drosomycin* and
669 *Transferrin1* that can be induced through either Toll or Imd (De Gregorio et al., 2002). Finally,
670 we observed rarer cases of synergy between Toll and Melanization, or Imd and Melanization.
671 Our study confirms that the Toll and Imd humoral modules have a defined roles against
672 broad classes of pathogens, Imd for Gram-negative bacteria and DAP-type containing Gram-
673 positive bacteria, and Toll for fungi and Gram-positive bacteria. Use of AMP and Bomanin
674 mutants revealed that this can be largely explained by the effectors they control. In contrast, the
675 contributions of Phagocytosis and Melanization appear to be critical to more specific and
676 diverse sets of pathogens. We speculate that Phagocytosis and/or Melanization have critical
677 roles in defense against bacteria that resist host defense peptides (Hanson et al., 2019), or can
678 hide from them (Touré et al., 2023). The melanization reaction is a source of ROS that is a
679 potent defense against pathogens such as *S. aureus* resistant to Toll and Imd defense (Dudzic
680 et al., 2019; Ford and King, 2021; Needham et al., 2004; Ramond et al., 2021). These two
681 modules play important roles in immune-related processes such as encapsulation
682 (Melanization), the uptake of bacteria escaping from the gut, or tissue homeostasis
683 (Phagocytosis) (Braun et al., 1998; C. Melcarne et al., 2019; Nehme et al., 2007) that were not
684 assessed here. Collectively, our study validates, with minor discrepancies, many studies that
685 have assessed the individual contributions of these modules (*e.g.* Apidianakis et al., 2005;
686 Binggeli et al., 2014; Lamiable et al., 2016; Lemaitre et al., 1996, 1995; Nehme et al., 2011).
687 Our mutant lines can now be used to analyze the contribution of these immune modules in

resistance to other pathogens, notably wasps, nematodes, microsporidia and protozoans, or in other contexts such as mating and local infection.

While defense against some pathogens is largely mediated by single immune modules, other pathogens are handled by multiple modules. We hypothesize that pathogens that have intermediate virulence levels (kill only a fraction of wild-type flies), may better reveal the roles of multiple modules. Indeed, stochasticity in survival analyses partly stems from the arms-race occurring between the pathogen and host immunity, as shown for *P. rettgeri* (Duneau et al., 2017). In this scenario, any small change in immune competence may tip the outcome of the arms-race toward lethality or survival. Here, it is notable that multiple modules contribute to survival in *P. rettgeri* infection. Previous studies have revealed a major role of the Imd pathway AMP Diptericin against this bacterium (Hanson et al., 2023, 2019; Unckless et al., 2016). However, Duneau et al. (Duneau et al., 2017) showed that survival patterns to *P. rettgeri* bifurcate into two outcomes based on time taken to fully activate systemic defenses, and showed a role for a Toll-PO SP cascade regulating serine proteases in defense against this microbe (Duneau et al., 2017). We may speculate that Melanization, although not as great a contributor as Diptericin, may be a factor that tips the balance of this precarious arms race towards host lethality. The observation that *ΔPhag* flies expressed less *DptA* (**Figure 1**) can explain their susceptibility to *P. rettgeri*. Future studies should investigate how multiple modules can contribute to host survival according to additive or Achilles dynamics – the concept that microbes have specific weaknesses available for host effectors to target (Hanson, 2024). It will also be important to consider the distinct roles of resistance, tolerance, and resilience in host defense (Howick and Lazzaro, 2017; Wukitch et al., 2023).

We observed a good correlation between survival analysis and pathogen growth in single module flies for *P. rettgeri*, *S. aureus,* and *C. albicans*. This indicates that these immune modules mostly contribute to resistance mechanisms that suppress pathogen growth. Our study did not reveal key early contributions of phagocytosis or melanization in pathogen growth control, despite quasi-immediate activation of these modules; higher *S. aureus* growth at 2 hours in *ΔPhag* flies was paralleled by *ΔIMD* flies, and we observed reduced Imd activation in *ΔPhag* flies. Melanization, while critical to surviving *S. aureus* infection, impacts bacterial growth beginning only at the 12-hour time point. This indicates that the microbicidal activity associated with Melanization in vivo acts more slowly than the blackening reaction observed in bled hemolymph. Interestingly, Δ*Phag* and also Δ*Mel* flies suppress *C. albicans* yeast growth, but some individuals ultimately succumb to infection with lower PLUD levels. We confirmed Δ*Phag* PLUD results using both an isogenic and a second wild-type genetic background, suggesting that this lower PLUD is genuine. Susceptibility to fungal infection independent of fungal proliferation has also been reported using an *A. fumigatus* septic infection model, and relies on the protection offered by Bomanins from pathogen-secreted toxins (Xu et al., 2023). It is tempting to speculate, based on the modules involved, that the loss of tolerance to *C. albicans* we observe is related to wound repair or accumulating damage, similar to reports of renal failure in beetles (Khan et al., 2017; Li et al., 2020), or flies with autotoxic trachea degradation upon stress pathway disruption (Rommelaere et al., 2024). Thus, this tolerance effect could rely on pathogen-mediated or autotoxic damage, which may be elucidated in a future study.

Here we have provided a single and double mutant analysis of *Drosophila* immune module functions. Our study provides several insights showing which modules are most important to survive infection by defined pathogens. However, we also highlight collective contribution of modules to defense, even when one module is of outsized importance. We extend our comprehension of innate immune responses by revealing higher complexity in defense, implicating multiple host immune modules in survival to various germs, including some with more cryptic contributions. As illustrated by our previous characterizations of AMP function (Carboni et al., 2022; Hanson et al., 2019), the melanization response (Dudzic et al., 2015), and stress-induced Turandot proteins (Rommelaere et al., 2024), a combinatorial mutation approach to deciphering immune function can be extended even to the broad level of whole immune modules. Together with these previous studies and others, our study provides an immunity toolkit, including flies simultaneously deficient for four immune modules, which can be used to better characterize the contributions of immune processes to host defense. Our tools can also be of use to study various questions at the cutting edge of aging, memory, neurodegeneration, cancer, and more, where immune genes are repeatedly implicated. We hope that this set of lines will be useful to the community to better characterize the *Drosophila* host defense.

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References

Ai X, Deng H, Li X, Wei Z, Chen Y, Yin T, Zhang J, Huang J, Li H, Lin X, Tan L, Chen D, Zhang Xiaohan, Zhang Xiuqing, Meignin C, Imler J-L, Cai H. 2024. cGAS-like receptors drive a systemic STING-dependent host response in Drosophila. *Cell*

775 *Reports* **43**:115081. doi:10.1016/j.celrep.2024.115081
776 Apidianakis Y, Mindrinos MN, Xiao W, Lau GW, Baldini RL, Davis RW, Rahme LG. 2005.
777 Profiling early infection responses: Pseudomonas aeruginosa eludes host defenses by
778 suppressing antimicrobial peptide gene expression. *Proc Natl Acad Sci U S A*
779 **102**:2573–8.
780 Binggeli O, Neyen C, Poidevin M, Lemaitre B. 2014. Prophenoloxidase Activation Is
781 Required for Survival to Microbial Infections in Drosophila. *PLoS Pathogens* **10**.
782 doi:10.1371/journal.ppat.1004067
783 Braun A, Hoffmann JA, Meister M. 1998. Analysis of the Drosophila host defense in domino
784 mutant larvae, which are devoid of hemocytes. *Proc Natl Acad Sci U S A* **95**:14337–
785 42.
786 Bretscher AJ, Honti V, Binggeli O, Burri O, Poidevin M, Kurucz E, Zsamboki J, Ando I,
787 Lemaitre B. 2015. The Nimrod transmembrane receptor Eater is required for
788 hemocyte attachment to the sessile compartment in Drosophila melanogaster. *Biology*
789 *open* **4**:355–63. doi:10.1242/bio.201410595
790 Buchon N, Poidevin M, Kwon H-M, Guillou A, Sottas V, Lee B-L, Lemaitre B. 2009. A
791 single modular serine protease integrates signals from pattern-recognition receptors
792 upstream of the Drosophila Toll pathway. *Proc Natl Acad Sci U S A* **106**:12442–
793 12447. doi:10.1073/pnas.0901924106
794 Cai H, Meignin C, Imler J-L. 2022. cGAS-like receptor-mediated immunity: the insect
795 perspective. *Curr Opin Immunol* **74**:183–189. doi:10.1016/j.coi.2022.01.005
796 Capilla A, Karachentsev D, Patterson RA, Hermann A, Juarez MT, McGinnis W. 2017. Toll
797 pathway is required for wound-induced expression of barrier repair genes in the
798 Drosophila epidermis. *Proceedings of the National Academy of Sciences* **114**:E2682–
799 E2688. doi:10.1073/pnas.1613917114
800 Carboni AL, Hanson MA, Lindsay SA, Wasserman SA, Lemaitre B. 2022. Cecropins
801 contribute to *Drosophila* host defense against a subset of fungal and Gram-negative
802 bacterial infection. *Genetics* **220**. doi:10.1093/genetics/iyab188
803 Carvalho L, Jacinto A, Matova N. 2014. The Toll/NF-κB signaling pathway is required for
804 epidermal wound repair in Drosophila. *PNAS* **111**:E5373–E5382.
805 doi:10.1073/pnas.1408224111
806 Cerenius L, Lee BL, Söderhäll K. 2008. The proPO-system: pros and cons for its role in
807 invertebrate immunity. *Trends in Immunology* **29**:263–271.
808 doi:10.1016/j.it.2008.02.009
809 Charroux B, Royet J. 2009. Elimination of plasmatocytes by targeted apoptosis reveals their
810 role in multiple aspects of the Drosophila immune response. *Proc Natl Acad Sci U S A*
811 **106**:9797–802. doi:10.1073/pnas.0903971106
812 Choe K-M, Lee H, Anderson KV. 2005. Drosophila peptidoglycan recognition protein LC
813 (PGRP-LC) acts as a signal-transducing innate immune receptor. *Proceedings of the*
814 *National Academy of Sciences* **102**:1122–1126. doi:10.1073/pnas.0404952102
815 Clemmons AW, Lindsay SA, Wasserman SA. 2015. An Effector Peptide Family Required for
816 Drosophila Toll-Mediated Immunity. *PLoS Pathogens* **11**.
817 doi:10.1371/journal.ppat.1004876
818 Costa A, Jan E, Sarnow P, Schneider D. 2009. The Imd pathway is involved in antiviral

immune responses in Drosophila. *PLoS ONE* **4**:e7436. doi:10.1371/journal.pone.0007436

Danilova N. 2006. The evolution of immune mechanisms. *J Exp Zool* **306B**:496–520. doi:10.1002/jez.b.21102

De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. 2002. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. *Embo J* **21**:2568–79.

Defaye A, Evans I, Crozatier M, Wood W, Lemaitre B, Leulier F. 2009. Genetic Ablation of Drosophila Phagocytes Reveals Their Contribution to Both Development and Resistance to Bacterial Infection. *Journal of Innate Immunity* **1**:322–334. doi:10.1159/000210264

Dudzic JP, Hanson MA, Iatsenko I, Kondo S, Lemaitre B. 2019. More Than Black or White: Melanization and Toll Share Regulatory Serine Proteases in Drosophila. *Cell Reports* **27**:1050-1061.e3. doi:10.1016/j.celrep.2019.03.101

Dudzic JP, Kondo S, Ueda R, Bergman CM, Lemaitre B. 2015. Drosophila innate immunity: regional and functional specialization of prophenoloxidases. *BMC biology* **13**:81–97. doi:10.1186/s12915-015-0193-6

Duneau D, Ferdy J-B, Revah J, Kondolf H, Ortiz G, Lazzaro B, Buchon N. 2017. Stochastic variation in the initial phase of bacterial infection predicts the probability of survival in D. melanogaster. *eLife* **6**. doi:10.7554/eLife.28298

Duneau DF, Kondolf H, Im J, Ortiz G, Chow C, Fox M, Eugénio A, Revah J, Buchon N, Lazzaro B. 2017. The Toll pathway underlies host sexual dimorphism in resistance to both Gram-negative and Gram-positive bacteria in mated Drosophila. *BMC Biology* **15**. doi:10.1186/s12915-017-0466-3

Duneau D, Lafont PD, Lauzeral C, Parthuisot N, Faucher C, Jin X, Buchon N, Ferdy J-B. 2025. A within-host infection model to explore tolerance and resistance. *eLife* **14**:e104052. doi:10.7554/eLife.104052

Elrod-Erickson M, Mishra S, Schneider D. 2000. Interactions between the cellular and humoral immune responses in drosophila. *Curr Biol* **10**:781–4.

Fehlbaum P, Bulet P, Michaut L, Lagueux M, Broekaert WF, Hetru C, Hoffmann JA. 1994. Insect immunity: Septic injury of drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. *Journal of Biological Chemistry* **269**:33159–33163.

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* **11**.

Ferreira AG, Naylor H, Esteves SS, Pais IS, Martins NE, Teixeira L. 2014. The Toll-dorsal pathway is required for resistance to viral oral infection in Drosophila. *PLoS pathogens* **10**:e1004507. doi:10.1371/journal.ppat.1004507

Ford SA, King KC. 2021. In Vivo Microbial Coevolution Favors Host Protection and Plastic Downregulation of Immunity. *Molecular Biology and Evolution* **38**:1330–1338. doi:10.1093/molbev/msaa292

Garg A, Wu LP. 2014. *D rosophila* Rab14 mediates phagocytosis in the immune response to *S taphylococcus aureus*: Rab14 is critical for cellular immune responses to *S. aureus*.

863 *Cellular Microbiology* **16**:296–310. doi:10.1111/cmi.12220
864 Goto A, Okado K, Martins N, Cai H, Barbier V, Lamiable O, Troxler L, Santiago E, Kuhn L,
865 Paik D, Silverman N, Holleufer A, Hartmann R, Liu J, Peng T, Hoffmann JA,
866 Meignin C, Daeffler L, Imler JL. 2018. The Kinase IKKβ Regulates a STING- and
867 NF-κB-Dependent Antiviral Response Pathway in Drosophila. *Immunity* **49**:225-
868 234.e4. doi:10.1016/j.immuni.2018.07.013
869 Gottar M, Gobert V, Matskevich AA, Reichhart J-M, Wang C, Butt TM, Belvin M, Hoffmann
870 JA, Ferrandon D. 2006. Dual Detection of Fungal Infections in Drosophila via
871 Recognition of Glucans and Sensing of Virulence Factors. *Cell* **127**:1425–1437.
872 doi:10.1016/j.cell.2006.10.046
873 Haine ER, Moret Y, Siva-Jothy MT, Rolff J. 2008. Antimicrobial Defense and Persistent
874 Infection in Insects. *Science* **322**:1257–1259. doi:10.1126/science.1165265
875 Hanson MA. 2024. When the microbiome shapes the host: immune evolution implications for
876 infectious disease. *Phil Trans R Soc B* **379**:20230061. doi:10.1098/rstb.2023.0061
877 Hanson MA, Dostálová A, Ceroni C, Poidevin M, Kondo S, Lemaitre B. 2019. Synergy and
878 remarkable specificity of antimicrobial peptides in vivo using a systematic knockout
879 approach. *eLife* **8**. doi:10.7554/elife.44341
880 Hanson MA, Grollmus L, Lemaitre B. 2023. Ecology-relevant bacteria drive the evolution of
881 host antimicrobial peptides in Drosophila. *Science* **381**:eadg5725.
882 doi:10.1126/science.adg5725
883 Hanson MA, Lemaitre B. 2023. Antimicrobial peptides do not directly contribute to aging in
884 *Drosophila* , but improve lifespan by preventing dysbiosis. *Disease Models &*
885 *Mechanisms* **16**:dmm049965. doi:10.1242/dmm.049965
886 Howick VM, Lazzaro BP. 2017. The genetic architecture of defense as resistance to and
887 tolerance of bacterial infection in Drosophila melanogaster. *Molecular Ecology*
888 **26**:1533–1546. doi:10.1111/mec.14017
889 Huang Z, Kingsolver MB, Avadhanula V, Hardy RW. 2013. An Antiviral Role for
890 Antimicrobial Peptides during the Arthropod Response to Alphavirus Replication.
891 *Journal of Virology* **87**:4272–4280. doi:10.1128/jvi.03360-12
892 Iatsenko I, Kondo S, Mengin-Lecreulx D, Lemaitre B. 2016. PGRP-SD, an Extracellular
893 Pattern-Recognition Receptor, Enhances Peptidoglycan-Mediated Activation of the
894 Drosophila Imd Pathway. *Immunity* **45**:1013–1023.
895 doi:10.1016/j.immuni.2016.10.029
896 Imler J-L, Bulet P. 2005. Antimicrobial peptides in Drosophila: structures, activities and gene
897 regulation. *Chemical immunology and allergy* **86**:1–21. doi:10.1159/000086648
898 Ip Y. 1993. Dif, a dorsal-related gene that mediates an immune response in Drosophila. *Cell*
899 **75**:753–763. doi:10.1016/0092-8674(93)90495-C
900 Issa N, Guillaumot N, Lauret E, Matt N, Schaeffer-Reiss C, Van Dorsselaer A, Reichhart JM,
901 Veillard F. 2018. The Circulating Protease Persephone Is an Immune Sensor for
902 Microbial Proteolytic Activities Upstream of the Drosophila Toll Pathway. *Molecular*
903 *Cell* **69**:648-663.e7. doi:10.1016/j.molcel.2018.01.029
904 Kaneko T, Goldman WE, Mellroth P, Steiner H, Fukase K, Kusumoto S, Harley W, Fox A,
905 Golenbock D, Silverman N. 2004. Monomeric and polymeric gram-negative
906 peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway. *Immunity*

20:637–49.
Kaneko Takashi, Yano T, Aggarwal K, Lim J-H, Ueda K, Oshima Y, Peach C, Erturk-Hasdemir D, Goldman WE, Oh B-H, Kurata S, Silverman N. 2006. PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. *Nat Immunol* **7**:715–723. doi:10.1038/ni1356
Kaneko T., Yano T, Aggarwal K, Lim JH, Ueda K, Oshima Y, Peach C, Erturk-Hasdemir D, Goldman WE, Oh BH, Kurata S, Silverman N. 2006. PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. *Nat Immunol* **7**:715–23.
Khan I, Agashe D, Rolff J. 2017. Early-life inflammation, immune response and ageing. *Proceedings of the Royal Society B: Biological Sciences* **284**:20170125. doi:10.1098/rspb.2017.0125
Kocks C, Cho JH, Nehme N, Ulvila J, Pearson AM, Meister M, Strom C, Conto SL, Hetru C, Stuart LM, Stehle T, Hoffmann JA, Reichhart J-M, Ferrandon D, Rämet M, Ezekowitz RAB. 2005. Eater, a Transmembrane Protein Mediating Phagocytosis of Bacterial Pathogens in Drosophila. *Cell* **123**:335–346. doi:10.1016/j.cell.2005.08.034
Kurucz É, Márkus R, Zsámboki J, Folkl-Medzihradszky K, Darula Z, Vilmos P, Udvardy A, Krausz I, Lukacsovich T, Gateff E, Zettervall C-J, Hultmark D, Andó I. 2007. Nimrod, a Putative Phagocytosis Receptor with EGF Repeats in Drosophila Plasmatocytes. *Current Biology* **17**:649–654. doi:10.1016/j.cub.2007.02.041
Lafont PDM, Lauzeral C, Parthuisot N, Faucher C, Duneau D, Ferdy J-B. 2021. A within-host infection model to explore tolerance and resistance (preprint). Evolutionary Biology. doi:10.1101/2021.10.19.464998
Lamiable O, Arnold J, de Faria IJ da S, Olmo RP, Bergami F, Meignin C, Hoffmann JA, Marques JT, Imler J-L. 2016. Analysis of the Contribution of Hemocytes and Autophagy to Drosophila Antiviral Immunity. *Journal of Virology* **90**:5415–5426. doi:10.1128/JVI.00238-16
Lemaitre B, Kromer-Metzger E, Michaut L, Nicolas E, Meister M, Georgel P, Reichhart JM, Hoffmann JA. 1995. A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense. *Proc Natl Acad Sci U S A* **92**:9465–9469. doi:10.1073/pnas.92.21.9465
Lemaitre B, Nicolas E, Michaut L, Reichhart J-M, Hoffmann JA. 1996. The Dorsoventral Regulatory Gene Cassette spätzle/Toll/cactus Controls the Potent Antifungal Response in Drosophila Adults. *Cell* **86**:973–983. doi:10.1016/S0092-8674(00)80172-5
Lemaitre B, Reichhart JM, Hoffmann JA. 1997. Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proceedings of the National Academy of Sciences of the United States of America* **94**:14614–9. doi:10.1073/pnas.94.26.14614
Leulier F, Parquet C, Pili-Floury S, Ryu J-H, Caroff M, Lee W-J, Mengin-Lecreulx D, Lemaitre B. 2003. The Drosophila immune system detects bacteria through specific peptidoglycan recognition. *Nat Immunol* **4**:478–484. doi:10.1038/ni922
Leulier F, Rodriguez A, Khush RS, Abrams JM, Lemaitre B. 2000. The Drosophila caspase Dredd is required to resist gram-negative bacterial infection. *EMBO Rep* **1**:353–8. doi:10.1093/embo-reports/kvd073

951 Li X, Rommelaere S, Kondo S, Lemaitre B. 2020. Renal Purge of Hemolymphatic Lipids
952 Prevents the Accumulation of ROS-Induced Inflammatory Oxidized Lipids and
953 Protects Drosophila from Tissue Damage. *Immunity* **52**:374-387.e6.
954 doi:10.1016/j.immuni.2020.01.008
955 Liegeois S, Ferrandon D. 2022. Sensing microbial infections in the Drosophila melanogaster
956 genetic model organism. *Immunogenetics* **74**:35–62. doi:10.1007/s00251-021-01239-0
957 Ligoxygakis P, Pelte N, Ji C, Leclerc V, Duvic B, Belvin M, Jiang H, Hoffmann JA,
958 Reichhart JM. 2002. A serpin mutant links Toll activation to melanization in the host
959 defense of Drosophila. *Embo J* **21**:6330–7.
960 Ligoxygakis P, Roth S, Reichhart J-M. 2003. A Serpin Regulates Dorsal-Ventral Axis
961 Formation in the Drosophila Embryo. *Current Biology* **13**:2097–2102.
962 doi:10.1016/j.cub.2003.10.062
963 Lindsay SA, Lin SJH, Wasserman SA. 2018. Short-Form Bomanins Mediate Humoral
964 Immunity in Drosophila. *J Innate Immun* **10**:306–314. doi:10.1159/000489831
965 Marra A, Hanson MA, Kondo S, Erkosar B, Lemaitre B. 2021. Drosophila Antimicrobial
966 Peptides and Lysozymes Regulate Gut Microbiota Composition and Abundance. *mBio*
967 e0082421. doi:10.1128/mBio.00824-21
968 Martins NE, Faria VG, Teixeira L, Magalhães S, Sucena É. 2013. Host Adaptation Is
969 Contingent upon the Infection Route Taken by Pathogens. *PLoS Pathogens*
970 **9**:e1003601. doi:10.1371/journal.ppat.1003601
971 Medzhitov R, Schneider DS, Soares MP. 2012. Disease Tolerance as a Defense Strategy.
972 *Science* **335**:936–941. doi:10.1126/science.1214935
973 Melcarne C. 2020. Role of Nimrod receptors in the Drosophila melanogaster cellular immune
974 response. EPFL. doi:10.5075/epfl-thesis-9933
975 Melcarne C., Lemaitre B, Kurant E. 2019. Phagocytosis in Drosophila: From molecules and
976 cellular machinery to physiology. *Insect Biochemistry and Molecular Biology* **109**:1–
977 12. doi:10.1016/j.ibmb.2019.04.002
978 Melcarne C, Ramond E, Dudzic J, Bretscher A, Kurucz E, Andó I, Lemaitre B. 2019. Two
979 Nimrod receptors, NimC1 and Eater, synergistically contribute to bacterial
980 phagocytosis in *Drosophila melanogaster*. *The FEBS Journal*. doi:10.1111/febs.14857
981 Nakano S, Kashio S, Nishimura K, Takeishi A, Kosakamoto H, Obata F, Kuranaga E,
982 Chihara T, Yamauchi Y, Isobe T, Miura M. 2023. Damage sensing mediated by serine
983 proteases Hayan and Persephone for Toll pathway activation in apoptosis-deficient
984 flies. *PLoS Genet* **19**:e1010761. doi:10.1371/journal.pgen.1010761
985 Nakhleh J, El Moussawi L, Osta MA. 2017. The Melanization Response in Insect
986 ImmunityAdvances in Insect Physiology. Elsevier. pp. 83–109.
987 doi:10.1016/bs.aiip.2016.11.002
988 Nappi A, Poirié M, Carton Y. 2009. The role of melanization and cytotoxic by-products in the
989 cellular immune responses of Drosophila against parasitic wasps. *Adv Parasitol*
990 **70**:99–121. doi:10.1016/S0065-308X(09)70004-1
991 Needham AJ, Kibart M, Crossley H, Ingham PW, Foster SJ. 2004. Drosophila melanogaster
992 as a model host for Staphylococcus aureus infection. *Microbiology* **150**:2347–2355.
993 doi:10.1099/mic.0.27116-0
994 Nehme NT, Liégeois S, Kele B, Giammarinaro P, Pradel E, Hoffmann JA, Ewbank JJ,

995 Ferrandon D. 2007. A Model of Bacterial Intestinal Infections in Drosophila
996 melanogaster. *PLoS Pathogens* **3**:e173. doi:10.1371/journal.ppat.0030173
997 Nehme NT, Quintin J, Cho JH, Lee J, Lafarge M-C, Kocks C, Ferrandon D. 2011. Relative
998 Roles of the Cellular and Humoral Responses in the Drosophila Host Defense against
999 Three Gram-Positive Bacterial Infections. *PLOS ONE* **6**:e14743.
1000 doi:10.1371/journal.pone.0014743
1001 Pradeu T, Thomma BPHJ, Girardin SE, Lemaitre B. 2024. The conceptual foundations of
1002 innate immunity: Taking stock 30 years later. *Immunity* 613–31.
1003 Quintin J, Asmar J, Matskevich AA, Lafarge M-C, Ferrandon D. 2013. The Drosophila Toll
1004 Pathway Controls but Does Not Clear Candida glabrata Infections. *The Journal of*
1005 *Immunology* **190**:2818–2827. doi:10.4049/jimmunol.1201861
1006 Rämet M, Lanot R, Zachary D, Manfruelli P. 2002. JNK Signaling Pathway Is Required for
1007 Efficient Wound Healing in Drosophila. *Developmental Biology* **241**:145–156.
1008 doi:10.1006/dbio.2001.0502
1009 Ramond E, Jamet A, Ding X, Euphrasie D, Bouvier C, Lallemant L, He X, Arbibe L,
1010 Coureuil M, Charbit A. 2021. Reactive Oxygen Species-Dependent Innate Immune
1011 Mechanisms Control Methicillin-Resistant Staphylococcus aureus Virulence in the
1012 *Drosophila* Larval Model. *mBio* **12**:e00276-21. doi:10.1128/mBio.00276-21
1013 Rommelaere S, Carboni A, Bada Juarez JF, Boquete J-P, Abriata LA, Meireles FTP, Rukes
1014 V, Vincent C, Kondo S, Dionne MS, Dal Peraro M, Cao C, Lemaitre B. 2024. A
1015 humoral stress response protects *Drosophila* tissues from antimicrobial peptides.
1016 *Current Biology* 1–12. doi:10.1101/2023.07.24.550293
1017 Rutschmann S, Kilinc A, Ferrandon D. 2002. Cutting edge: the toll pathway is required for
1018 resistance to gram- positive bacterial infections in Drosophila. *J Immunol* **168**:1542–6.
1019 Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, Drummond J, Webster J,
1020 Gubb D, Gunton N, Johnson G, O'Kane CJ, Huen D, Sharma P, Asztalos Z, Baisch H,
1021 Schulze J, Kube M, Kittlaus K, Reuter G, Maroy P, Szidonya J, Rasmuson-Lestander
1022 \AAsa, Ekström K, Dickson B, Hugentobler C, Stocker H, Hafen E, Lepesant JA,
1023 Pflugfelder G, Heisenberg M, Mechler B, Serras F, Corominas M, Schneuwly S, Preat
1024 T, Roote J, Russell S. 2004. The DrosDel collection: A set of P-element insertions for
1025 generating custom chromosomal aberrations in Drosophila melanogaster. *Genetics*
1026 **167**:797–813. doi:10.1534/genetics.104.026658
1027 Sansone CL, Cohen J, Yasunaga A, Xu J, Osborn G, Subramanian H, Gold B, Buchon N,
1028 Cherry S. 2015. Microbiota-dependent priming of antiviral intestinal immunity in
1029 Drosophila. *Cell host & microbe* **18**:571–581.
1030 Schmid-Hempel P. 2021. Evolutionary parasitology: the integrated study of infections,
1031 immunology, ecology, and genetics. Oxford University Press.
1032 Shan T, Wang Y, Bhattarai K, Jiang H. 2023. An evolutionarily conserved serine protease
1033 network mediates melanization and Toll activation in *Drosophila*. *Sci Adv*
1034 **9**:eadk2756. doi:10.1126/sciadv.adk2756
1035 Stephenson HN, Streeck R, Grüblinger F, Goosmann C, Herzig A. 2022. Hemocytes are
1036 essential for *Drosophila melanogaster* post-embryonic development, independent of
1037 control of the microbiota. *Development* **149**:dev200286. doi:10.1242/dev.200286
1038 Touré H, Durand N, Guénal I, Herrmann J-L, Girard-Misguich F, Szuplewski S. 2023.

1039 Mycobacterium abscessus Opsonization Allows an Escape from the Defensin
1040 Bactericidal Action in *Drosophila*. *Microbiol Spectr* e00777-23.
1041 doi:10.1128/spectrum.00777-23
1042 Ulvila J, Vanha-Aho L-M, Rämet M. 2011. Drosophila phagocytosis - still many unknowns
1043 under the surface. *APMIS* **119**:651–662. doi:10.1111/j.1600-0463.2011.02792.x
1044 Unckless RL, Howick VM, Lazzaro BP. 2016. Convergent Balancing Selection on an
1045 Antimicrobial Peptide in Drosophila. *Current Biology* **26**:257–262.
1046 doi:10.1016/j.cub.2015.11.063
1047 Uttenweiler-Joseph S, Moniatte M, Lagueux M, Van Dorsselaer A, Hoffmann JA, Bulet P.
1048 1998. Differential display of peptides induced during the immune response of
1049 Drosophila: a matrix-assisted laser desorption ionization time-of- flight mass
1050 spectrometry study. *Proc Natl Acad Sci U S A* **95**:11342–7.
1051 Vaz F, Kounatidis I, Covas G, Parton RM, Harkiolaki M, Davis I, Filipe SR, Ligoxygakis P.
1052 2019. Accessibility to Peptidoglycan Is Important for the Recognition of Gram-
1053 Positive Bacteria in Drosophila. *Cell Reports* **27**:2480-2492.e6.
1054 doi:10.1016/j.celrep.2019.04.103
1055 Westlake H, Hanson MA, Lemaitre B. 2024. The Drosophila immunity handbook, First. ed.
1056 EPFL Press. doi:10.55430/6304TDIHVA01
1057 Wukitch AM, Lawrence MM, Satriale FP, Patel A, Ginder GM, Van Beek EJ, Gilani O,
1058 Chambers MC. 2023. Impact of Chronic Infection on Resistance and Tolerance to
1059 Secondary Infection in Drosophila melanogaster. *Infect Immun* e00360-22.
1060 doi:10.1128/iai.00360-22
1061 Xu R, Lou Y, Tidu A, Bulet P, Heinekamp T, Martin F, Brakhage A, Li Z, Liégeois S,
1062 Ferrandon D. 2023. The Toll pathway mediates Drosophila resilience to Aspergillus
1063 mycotoxins through specific Bomanins. *EMBO Rep* **24**:e56036.
1064 doi:10.15252/embr.202256036
1065 Yasin B, Wang W, Pang M, Cheshenko N, Hong T, Waring AJ, Herold BC, Wagar EA,
1066 Lehrer RI. 2004. θ Defensins Protect Cells from Infection by Herpes Simplex Virus by
1067 Inhibiting Viral Adhesion and Entry. *Journal of Virology* **78**:5147–5156.
1068 doi:10.1128/jvi.78.10.5147-5156.2004

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1070 ## Figure Legends

1071 **Table 1: List of mutants used in this study.** "-" indicates the deleted immune module.

1072

1073 **Figure 1: Each immune module functions largely independently from other immune**
1074 **modules**. **A)** Activation of the Imd pathway, as revealed by *DptA* expression following
1075 infection with a mixture of heat-killed *E. coli* and *M. luteus,* is broadly wild-type (WT) in single
1076 module mutants other than Imd. In the case of *ΔPhag* flies, we observed a lesser induction at
1077 6h compared to wild-type. **B)** Activation of the Toll pathway as revealed by the expression of
1078 *Drs* is broadly wild-type in single module mutants other than *ΔTOLL* flies. Note that *Drs*
1079 receives a minor input from Imd signaling (Leulier et al., 2000), explaining minor induction of
1080 *Drs* in *ΔTOLL* flies at early time points. In the case of *ΔMel*, we observed a slightly greater Toll
1081 pathway activity. **C)** The ability of plasmatocytes to phagocytose bacterial particles is not

negatively affected in single module mutants except *ΔPhag* (also see Figure 1supp1) **D)** Cuticle blackening after clean injury is not impaired except in *ΔMel* flies. **E)** Activation of the Imd pathway remains strongly inducible in compound mutants except when deficient for the Imd pathway. **F)** Activation of the Toll pathway remains strongly inducible in compound mutants except when flies were deficient of the Toll pathway. **G)** The ability of plasmatocytes to phagocytose bacterial particles is not significantly affected in compound mutants except those including *ΔPhag*. **H)** Cuticle blackening after clean injury is not impaired in compound mutants except in those including *ΔMel*.

Figure 2: Lifespans of mutants used in this study in unchallenged flies and upon clean injury conditions at 25°C. **A-B)** Single module mutants; **C-D)** compound module mutants. See Figure 2supp1 for 29°C comparisons.

Figure 3: Heatmap of lifespans of immune module-deficient flies upon infection by various pathogens. Darker blue indicates lower survival, while white indicates maximum survival. Experiments used either 7 or 10 days as a maximum lifespan/time course, and the heatmap is adjusted accordingly per row to have white fill for the maximum possible lifespan of that row. Small text to right of mean lifespan indicates total flies. Survival curves are presented in **Supplementary file 1**, and a summary of susceptibilities is provided in Figure 3supp1.

Figure 4: Growth kinetics of *P. rettgeri* (A), *S. aureus* (B), and *C. albicans* (C) in wild-type, single module mutant and *ΔITPM* flies. Survival curves from **Supplementary file 1** underlying data in **Figure 3** are shown for context. Each data point reflects a pooled sample of 5 flies.

Figure 5: Measurement of PLUD upon infection with *P. rettgeri*, *S. aureus*, and *C. albicans*. A) The PLUD of *P. rettgeri* in individual module mutant flies is not significantly different from wild-type. The PLUD of *ΔITPM* flies was not significantly different, although censoring of a single low-PLUD outlier in the wild-type would result in a significant difference between wild-type and *ΔITPM* flies ($p < .01$). **B)** The PLUD of *S. aureus*-infected flies is not different across any genotype. **C)** The PLUD of *C. albicans*-infected flies was significantly lower in *ΔPhag* flies with a notably lower mean PLUD. This difference was robust to use of different *ΔPhag* genetic backgrounds (merged data shown here). *ΔMel* flies and *ΔITPM* flies also had significantly lower PLUD. *, $P < 0.05$; **, $P < 0.01$

Table S1: Microbe characteristics and growth conditions.

Figure 1supp1: We used *ΔPhag* mutants in the genetic background of Melcarne et al. (2019) *(+; ΔPhag)* in some experiments, and confirm here that these mutants are equally deficient in phagocytic capacity to *ΔPhag* flies in the iso DrosDel genetic background (*ΔPhag)*.

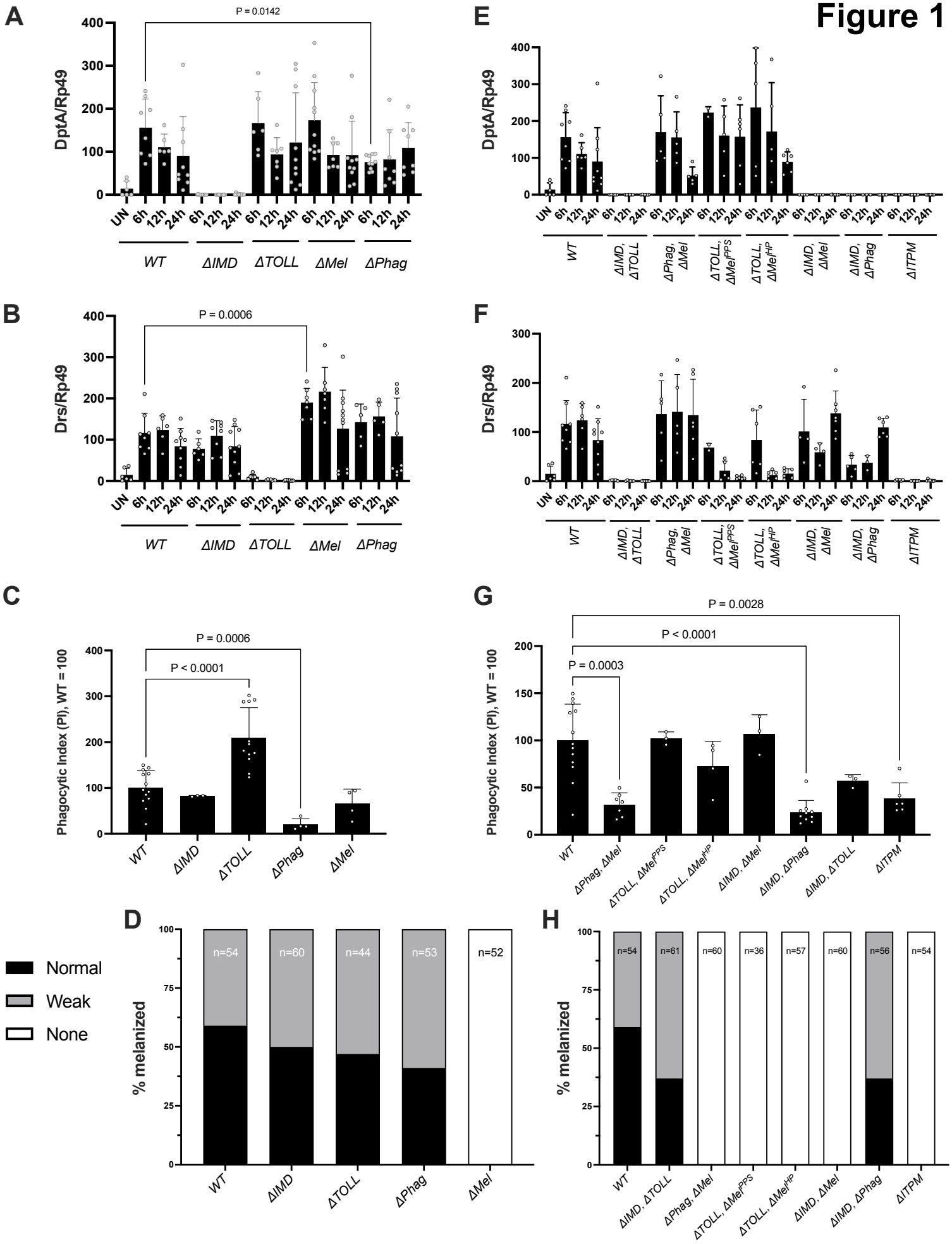
Figure 2supp1: Lifespans of mutants used in this study in unchallenged flies and upon clean injury conditions at 29°C. **A-B)** Single module mutants; **C-D)** compound module mutants.

1125
1126 **Supplementary file 1:** Survival curves for all data summarized in **Figure 3**.
1127
1128 **Figure 3supp1:** Summary table of susceptibilities per the three conditions outlined in section
1129 "Systemic infections and survival." ✓ indicates that the pathway contributes to defense against
1130 a given pathogen. In the event that a single module mutant is not more susceptible to the
1131 infection, but contributes to increased mortality in a double mutant line, a ✓ is given and the
1132 co-deleted relevant pathways highlighted by their first letter. Black boxes indicate no difference
1133 to wild-type or to clean injury. NI, natural infection; SI, septic infection.

			Deficient pathway			
Drosophila lines	**Reference**	**Name**	**IMD**	**Toll**	**Phagocytosis**	**Melanization**
iso ; iso ; Rel^{E20}	Hedengren et al., 1999	Δ*IMD*	-	+	+	+
iso ; iso ; spz^{rm7}	Lemaitre et al., 1996	Δ*TOLL*	+	-	+	+
iso ; NimC1^{1} *; Eater*^{1}	Melcarne et al., 2019	Δ*Phag*	+	+	-	+
*iso ; PPO1*Δ*, PPO2*Δ*; iso*	Bingelli et al., 2014	Δ*Mel*	+	+	+	-
+ ; + ; Rel^{E20}*, spz*^{rm7}	this paper	Δ*IMD,* Δ*TOLL*	-	-	+	+
*iso ; PPO1*Δ*, PPO2*Δ *; NimC1*^{1}*,Eater*^{1}	this paper	Δ*Phag,* Δ*Mel*	+	+	-	-
iso ; NimC1^{1} *; Eater*^{1}*, Rel*^{E20}	this paper	Δ*IMD,* Δ*Phag*	-	+	-	+
*iso ; PPO1*Δ *PPO2*Δ *; Rel*^{E20}	this paper	Δ*IMD,* Δ*Mel*	-	+	+	-
*iso ; PPO1*Δ *PPO2*Δ *; spz*^{rm7}	this paper	Δ*TOLL,* Δ*Mel*^{PPS}	+	-	+	-
iso Hay-psh^{Def} *; iso ; iso*	Dudzic et al., 2019	Δ*TOLL,* Δ*Mel*^{HP}	+	-	+	-
iso Hay-psh^{Def} *; NimC1*^{1} *; Eater*^{1} *Rel*^{E20}	this paper	Δ*ITPM*	-	-	-	-

Table 1

Figure 1


A
P = 0.0142
DptA/Rp49
UN
6h
12h
24h
WT
ΔIMD
ΔTOLL
ΔMel
ΔPhag
B
P = 0.0006
Drs/Rp49
C
P = 0.0006
P < 0.0001
Phagocytic Index (PI), WT = 100
D
Normal
Weak
None
% melanized
n=54
n=60
n=44
n=53
n=52
E
DptA/Rp49
ΔIMD, ΔTOLL
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔITPM
F
Drs/Rp49
G
P = 0.0028
P < 0.0001
P = 0.0003
Phagocytic Index (PI), WT = 100
H
% melanized
n=54
n=61
n=60
n=36
n=57
n=60
n=56
n=54

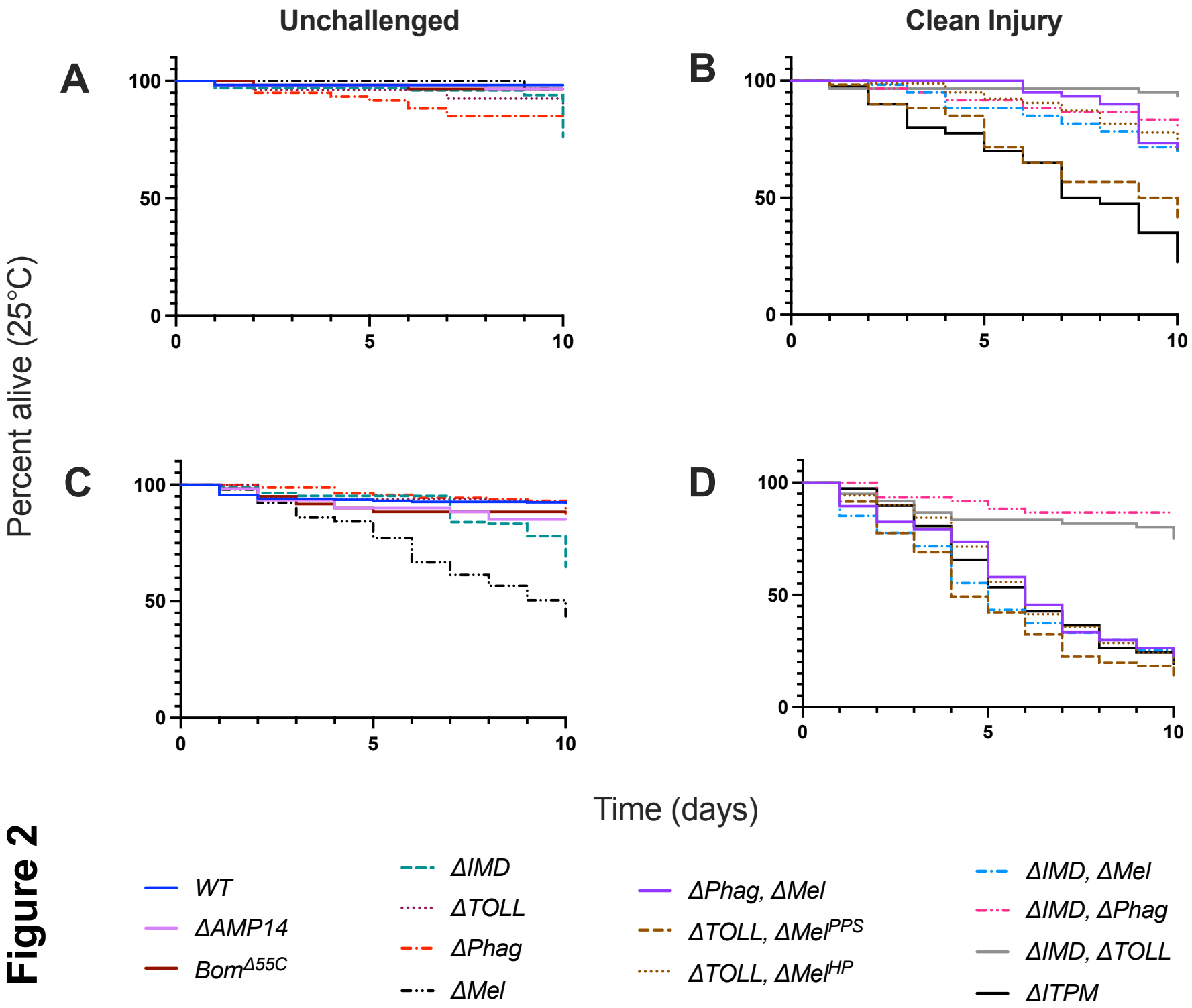

Unchallenged
Clean Injury
A
B
C
D
Percent alive (25°C)
Time (days)
100
50
0
0
5
10
WT
ΔAMP14
Bom^Δ55C
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM

Figure 2

Type	Species	Temp	Dose	Days	N exp	*WT*		*ΔAMP14*		*ΔBom*		*ΔIMD*		*ΔTOLL*		*ΔPhag*		*ΔMel*		*ΔPhag, ΔMel*		*ΔTOLL, ΔMel^{PPS}*		*ΔTOLL, ΔMel^{HP}*		*ΔIMD, ΔMel*		*ΔIMD, ΔPhag*		*ΔIMD, ΔTOLL*		*ΔITPM*	
Controls	*Unchallenged*	25°C	n/a	7	13	6.9	177	6.9	60	6.9	60	6.8	60	6.8	80	7.0	60	6.6	60	6.6	60	7.0	116	6.8	239	6.0	60	6.6	60	6.8	100	5.8	80
	Clean Injury	25°C	n/a	7	36	6.7	709	6.6	60	6.5	60	6.7	543	6.6	260	6.8	248	6.4	526	5.3	57	4.6	71	5.4	70	4.7	67	6.5	60	6.2	60	5.5	445
	Clean Injury	29°C	n/a	7	11	6.6	211	6.5	60	5.9	60	6.3	100	6.1	75	6.6	60	6.4	119	4.9	96	3.0	67	4.8	56	3.7	57	6.0	64	5.6	60	3.9	94
	Clean Injury	25°C	n/a	10	36	9.6	709	9.1	60	9.2	60	9.2	543	9.3	260	9.6	248	8.5	526	6.2	57	5.2	71	6.3	70	5.6	67	9.1	60	8.7	60	6.3	445
	Unchallenged	29°C	n/a	10	11	9.9	139	9.3	60	9.5	60	9.4	49	7.1	34	8.7	60	8.5	60	9.1	60	9.2	60	9.3	40	5.9	64	9.3	40	8.9	60	7.7	61
Viruses	*Drosophila C virus*	25°C	2000 TCID50	10	5	8.3	100	6.7	89	7.7	85	6.8	73	7.8	83	8.1	78	6.3	86	6.3	88	5.5	56	5.0	92	5.1	60	6.7	64	3.8	53	4.1	78
	Flock House virus	25°C	250000 TCID50	10	5	8.5	122	7.1	73	7.2	84	7.3	79	7.3	77	7.3	81	6.9	82	6.2	82	5.1	56	6.0	85	4.9	61	6.8	73	5.2	59	5.1	84
	Drosophila X virus	25°C	431000 TCID50	10	2	9.0	40	6.9	41	8.2	20	7.1	45	7.9	34	7.8	41	5.4	38	7.4	46	4.9	37	5.1	38	6.0	15	6.2	40	5.6	19	4.6	43
	Invertebrate iridescent-6 virus	25°C	31700 TCID50	10	3	9.4	59	7.5	50	9.4	64	8.1	58	8.4	54	8.8	60	5.6	58	6.0	61	5.8	59	5.8	58	6.1	48	8.3	54	5.9	47	4.4	69
	Sindbis virus	25°C	50000 TCID50	10	3	9.4	58	8.4	61	8.6	61	8.9	60	8.8	60	8.3	60	5.4	65	5.9	66	4.9	51	4.8	59	4.5	60	7.0	50	4.5	45	4.3	61
Fungi	*Aspergillus fumigatus* (natural)	29°C	rolled on plate	10	5	7.0	80	6.8	81	7.0	20	6.8	56	6.5	81	6.8	41	6.7	60	6.5	60	5.7	62	6.5	60	6.0	60	6.5	66	5.9	60	4.5	92
	Beauvaria bassiana (natural)	29°C	30mg	10	5	9.6	120	8.2	61	9.7	60	9.4	60	7.3	70	7.8	43	8.7	76	7.7	60	6.3	64	6.0	36	7.3	66	7.1	40	6.5	51	4.4	62
	Beauvaria bassiana	29°C	OD=5	7	6	6.4	81	6.0	74	3.6	59	5.7	58	3.6	66	5.2	39	5.3	62	4.4	57	2.6	59	3.4	63	4.4	21	4.7	51	3.1	56	3.3	95
	Candida albicans	29°C	OD=200	7	8	6.7	160	5.6	45	6.0	102	5.9	94	5.0	89	6.4	65	5.9	60	6.4	102	1.7	20	3.1	156	5.7	36	5.9	89	3.2	27	2.4	82
Gram positive bacteria	*Mycobacterium marinum*	25°C	OD=1	7	6	6.1	132	6.0	50	6.5	51	6.5	111	5.9	108	5.2	77	5.8	99	5.7	108	4.1	62	5.8	84	5.1	51	5.1	64	6.3	56	3.9	117
	Corynebacterium diphteriae	25°C	OD=100	7	3	6.0	73	6.2	84	6.5	97	5.6	79	5.1	80	6.6	87	4.6	82	5.8	69	3.2	47	3.3	103	4.9	72	5.9	88	4.5	61	3.0	75
	Micrococcus luteus	29°C	OD=200	7	8	6.7	121	5.7	80	6.8	60	6.2	120	6.4	148	6.5	71	6.5	81	5.6	81	4.6	68	6.5	80	4.8	77	6.3	84	2.1	102	2.1	91
	Streptococcus pneumoniae	25°C	OD=0.3	7	3	6.8	41	6.1	60	6.4	60	6.7	61	6.6	70	6.3	60	6.2	60	6.4	60	5.7	60	5.0	61	5.1	60	6.0	60	5.5	60	4.5	50
	Enterococcus faecalis	25°C	OD=5	7	10	5.0	130	4.5	100	2.1	62	4.2	122	1.8	126	5.2	79	5.1	122	3.9	76	1.6	63	1.8	102	4.2	62	4.0	79	1.4	61	1.3	101
	Staphylococcus aureus	25°C	OD=0.5	7	8	5.1	119	4.8	58	5.7	21	4.2	118	3.4	73	3.6	79	2.4	73	1.9	78	2.0	80	2.0	112	2.1	78	4.1	59	3.4	40	1.9	87
	Listeria monocytogenes	25°C	OD=0.5	7	5	4.8	91	4.7	78	2.2	60	2.3	62	1.6	112	4.6	71	5.3	76	3.9	78	2.1	70	2.2	73	3.5	88	3.2	60	2.0	62	1.6	62
	Bacillus subtilis	25°C	OD=5	7	7	5.7	120	3.3	40	4.1	20	4.0	123	3.8	119	6.0	55	5.4	110	4.5	98	2.8	69	2.7	84	4.5	76	5.4	20	2.1	41	2.0	81
Gram negative bacteria	*Vibrio parahemolyticus*	25°C	OD=10	7	2	3.1	58	1.1	61	2.9	62	1.1	62	1.3	70	2.8	64	2.3	70	3.2	57	1.8	68	1.9	41	1.0	59	1.1	63	1.0	58	1.0	64
	Providencia rettgeri	25°C	OD=0.1	7	7	5.5	146	1.1	57	5.1	58	2.0	78	4.4	70	3.6	100	1.8	95	1.5	61	1.5	75	5.3	40	1.3	75	2.1	62	1.0	21	1.4	123
	Providencia burhodogranaria	25°C	OD=10	7	7	5.1	161	1.7	76	4.6	40	1.6	58	4.6	88	4.9	63	4.0	124	4.2	84	2.9	61	4.9	60	1.3	78	1.0	61	1.4	40	1.3	68
	Pectobacterium carotovorum Ecc15	29°C	OD=10	7	7	6.4	131	1.5	57	4.8	20	1.9	91	5.8	108	4.4	120	5.8	100	5.2	102	4.7	60	5.2	60	1.8	72	-	-	1.4	62	1.4	106
	Klebsiella pneumoniae	25°C	OD=1	7	3	6.8	60	1.4	68	6.5	60	1.5	77	6.8	60	6.9	60	5.8	60	6.3	60	3.9	80	6.9	60	1.3	59	2.0	56	-	-	1.3	64
	Enterobacter cloacae	25°C	OD=10	7	8	6.6	149	1.3	83	6.7	60	1.0	100	6.6	90	6.7	60	6.4	80	6.1	80	4.9	61	5.8	99	1.3	73	1.0	37	1.6	61	1.2	83
	Escherichia coli	25°C	OD=200	7	8	6.6	141	5.0	79	6.9	40	3.8	80	6.2	70	5.4	20	5.5	43	5.5	85	3.9	63	5.3	63	3.8	60	4.6	60	1.7	70	1.6	60
	Salmonella enterica ser. typhimurium	25°C	OD=200	7	4	6.7	80	4.8	40	6.6	80	5.1	60	6.3	40	6.7	20	6.3	40	6.6	63	5.5	40	4.8	64	4.7	61	3.9	18	2.7	56	1.8	68

Figure 3

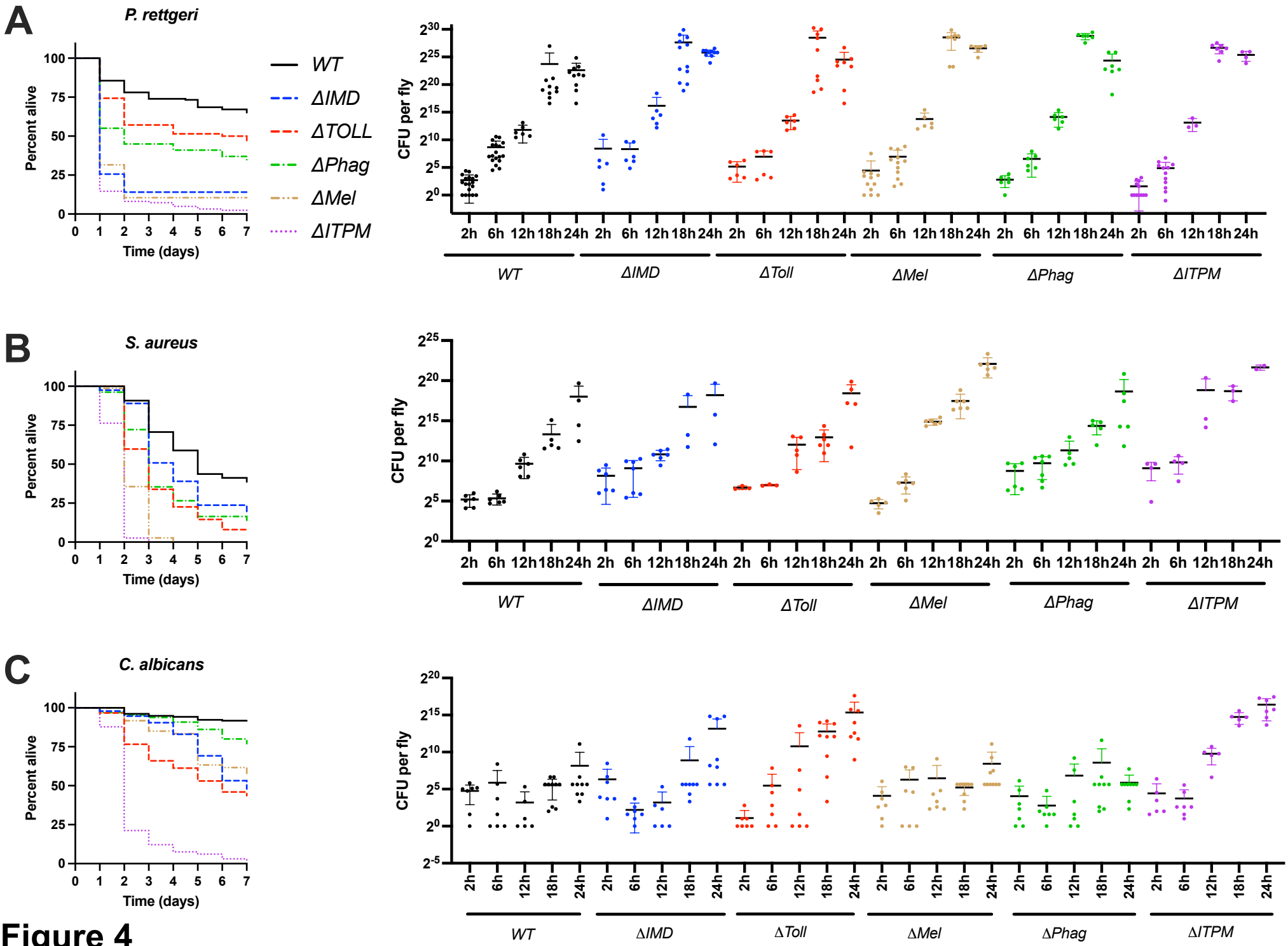

A
P. rettgeri
Percent alive
Time (days)
WT
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔITPM
CFU per fly
2h 6h 12h 18h 24h
WT
ΔIMD
ΔToll
ΔMel
ΔPhag
ΔITPM
B
S. aureus
C
C. albicans

Figure 4

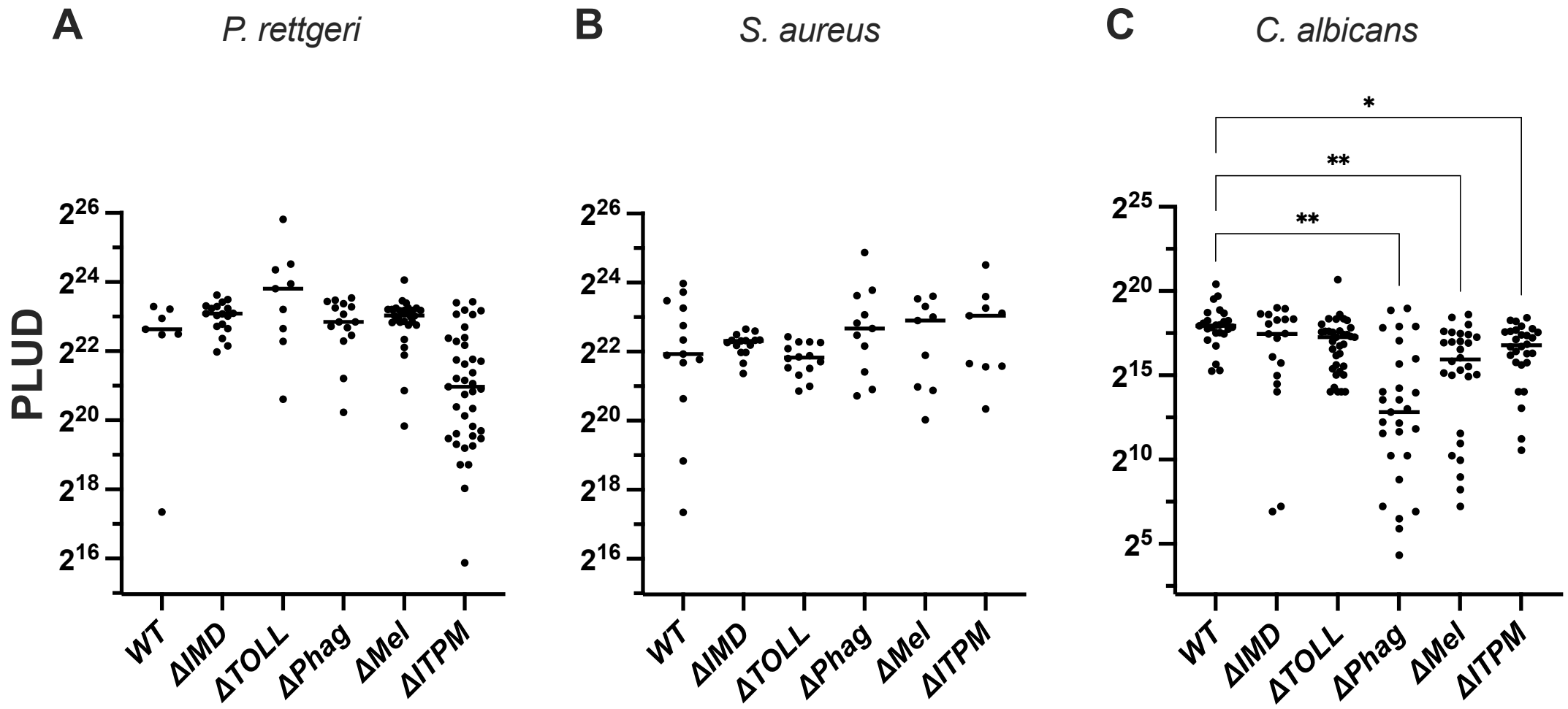

A
P. rettgeri
B
S. aureus
C
C. albicans
PLUD
2^{26}
2^{24}
2^{22}
2^{20}
2^{18}
2^{16}
2^{25}
2^{15}
2^{10}
2^{5}
*
**
**
WT
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔITPM

Figure 5

Type	Kingdom	Phylum	Class	Order	Family	Nucleic acid / Membrane	Species	Strain, Source, DOI of previous study	Inf. mode	Infection temperature	Used concentration	Growth temperature	Medium of culture
Viruses	Bamfordvirae	Nucleocytoviricota	Megaviricetes	Pimascovirales	Iridoviridae	dsDNA+	***Invertebrate iridescent-6 virus***	gift from Ronald van Rij	intra-thoracique	25°C	31 700 TCID50	provided	provided
	Orthornavirae	nd	nd	nd	Bimaviridae	ssRNA+	***Drosophila X virus***	gift from Ronald van Rij	intra-thoracique	25°C	431 000 TCID50	provided	provided
	Orthornavirae	Pisuviricota	Pisoniviricetes	Picornavirales	Dicistroviridae	ssRNA+	***Drosophila C virus***	gift from Carla Saleh	intra-thoracique	25°C	2 000 TCID50	26°C	S2 cells in Schneider
	Orthornavirae	Kitrinoviricota	Magsaviricetes	Nodamuvirales	Nodaviridae	dsRNA+	***Flock House virus***	gift from Carla Saleh	intra-thoracique	25°C	250 000 TCID50	26°C	S2 cells in Schneider
	Orthornavirae	Kitrinoviricota	Alsuviricetes	Martelliirales	Togaviridae	ssRNA+	***Sindbis virus***	gift from Ronald van Rij	intra-thoracique	25°C	50 000 TCID50	provided	provided
Fungi	Fungi	Ascomycota	Sacharomycete	Sacharomycetales	Sacharomycetacae	*Beta-glucans*	***Candida albicans***	ATCC 2001	septic	29°C	OD^{600}= 200	37°C	YPG
		Ascomycota	Eurotiomycete	Eurotiales	Trychocomacae	*Beta-glucans*	***Aspergillus fumigatus***	*doi: 10.7554/eLife.44341*	natural	29°C	rolled on plate	37°C	Malt-agar
		Ascomycota	Sordaryomycete	Coricypitacae	Ophiocordycipitacae	*Beta-glucans*	***Beauvaria bassiana***		septic	29°C	OD^{600}= 5	commercial	commercial
						Beta-glucans		R444	natural	29°C	30mg	commercial	commercial
Gram positive bacteria	Bacteria	Actinomycetota	Actinomycetia	Mycobacteriales	Mycobacteriacae	mAGP-type	***Mycobacterium marinum***	1218R WT	intra-thoracique	25°C	OD^{600}= 1	37°C	LB
		Actinomycetota	Actinomycetia	Mycobacteriales	Corynebacteriaceae	Lys-type	***Corynebacterium diphtheriae***	*DSM 44123*	septic	25°C	OD^{600}= 100	37°C	LB
		Actinomycetota	Actinomycetia	Actinomycetales	Micrococcacae	Lys-type	***Micrococcus luteus***	*doi: 10.7554/eLife.44341*	septic	25°C	OD^{600}= 200	29°C	LB
		Bacillota	Bacilli	Lactobacilliales	Streptococcacae	Lys-type	***Streptococcus pneumoniae***	D39V WT	septic	25°C	OD^{600}= 0.3	37°C	LB
		Bacillota	Bacilli	Lactobacilliales	Enterococcacae	Lys-type	***Entecoccus faecalis***	254	septic	25°C	OD^{600}= 5	37°C	LB
		Bacillota	Bacilli	Caryophanale	Staphylococcacae	Lys-type	***Staphylococcus aureus***	*doi: 10.7554/eLife.44341*	septic	25°C	OD^{600}= 0.5	37°C	BHI
		Bacillota	Bacilli	Bacilliales	Listeriacae	DAP-type	***Listeria monocytogenes***	BUG 1600	septic	25°C	OD^{600}= 0.5	37°C	LB
		Bacillota	Bacilli	Bacilliales	Bacillacae	DAP-type	***Bacillus subtilis***	168	septic	25°C	OD^{600}= 5	37°C	BHI
Gram negative bacteria	Bacteria	Pseudomonadota	Gammaproteobacteriacae	Pseudomonadales	Pseudomonadacae	DAP-type	***Pseudomonas aeruginosa***	PAO1	septic	25°C	OD^{600}= 0.01	37°C	LB
		Pseudomonadota	Gammaproteobacteriacae	Vibrionales	Vibrionacae	DAP-type	***Vibrio parahemolyticus***		septic	25°C	OD^{600}= 10	37°C	LB
		Pseudomonadota	Gammaproteobacteriacae	Enterobacteriales	Morgenallacae	DAP-type	***Providencia rettgeri***	Dmel	septic	25°C	OD^{600}= 0.1	37°C	LB
		Pseudomonadota	Gammaproteobacteriacae	Enterobacteriales	Morgenallacae	DAP-type	***Providencia burhodogranaria***	B	septic	25°C	OD^{600}= 10	37°C	LB
		Pseudomonadota	Gammaproteobacteriacae	Enterobacteriales	Enterobacteriacae	DAP-type	***Pectobacterium carotovorum***	Ecc15	septic	25°C	OD^{600}= 10	37°C	LB
		Pseudomonadota	Gammaproteobacteriacae	Enterobacteriales	Enterobacteriacae	DAP-type	***Klebsiella pneumoniae***		septic	25°C	OD^{600}= 1.0	37°C	LB
		Pseudomonadota	Gammaproteobacteriacae	Enterobacteriales	Enterobacteriacae	DAP-type	***Enterobacter cloacae***	B12	septic	25°C	OD^{600}= 10	37°C	LB
		Pseudomonadota	Gammaproteobacteriacae	Enterobacteriales	Enterobacteriacae	DAP-type	***Escherichia coli***	1106	septic	25°C	OD^{600}= 200	37°C	LB
		Pseudomonadota	Gammaproteobacteriacae	Enterobacteriales	Enterobacteriacae	DAP-type	***Salmonella enterica ser. typhimurium***	*doi: 10.7554/eLife.44341*	septic	25°C	OD^{600}= 200	37°C	LB

Table S1

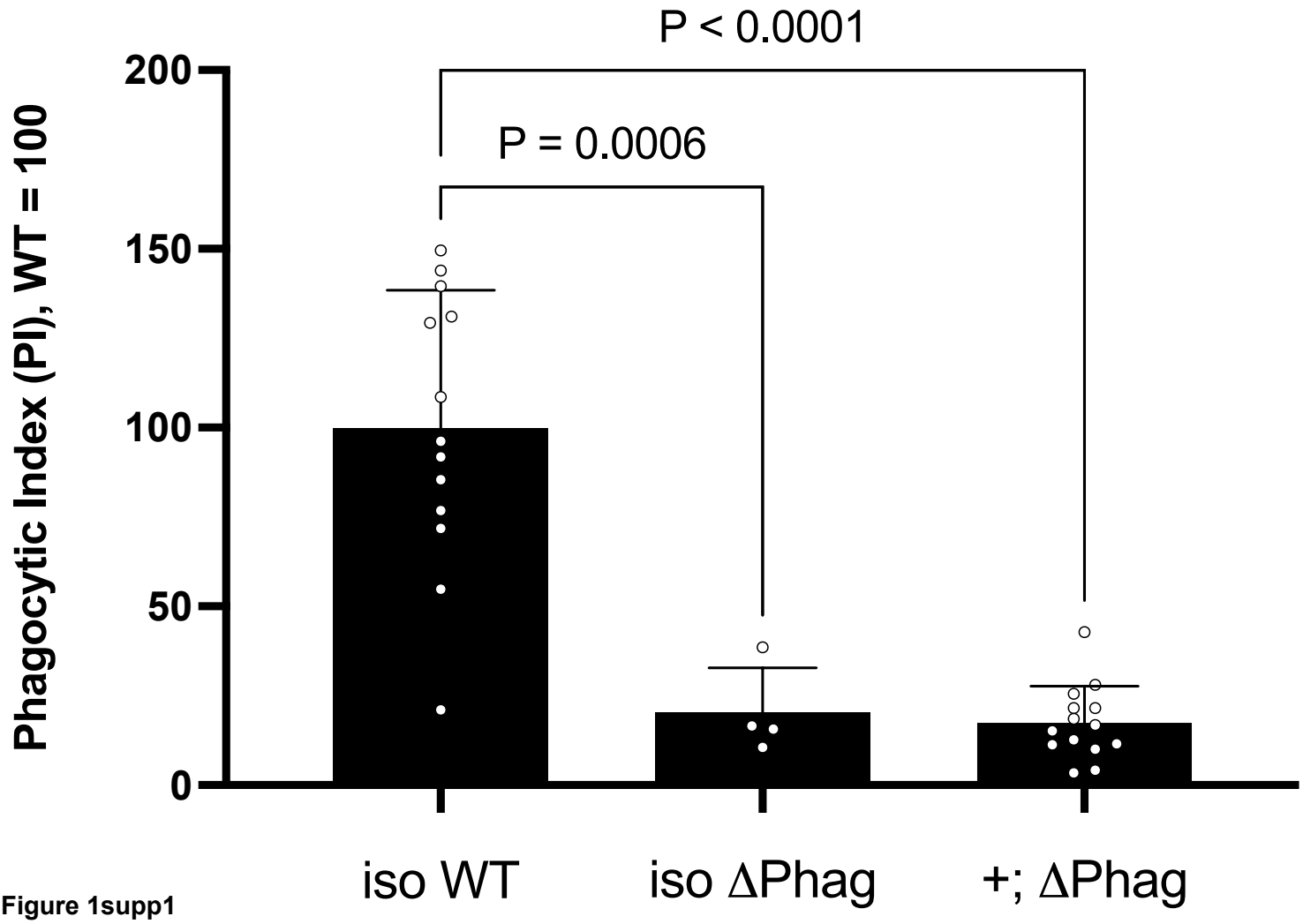


Figure 1supp1

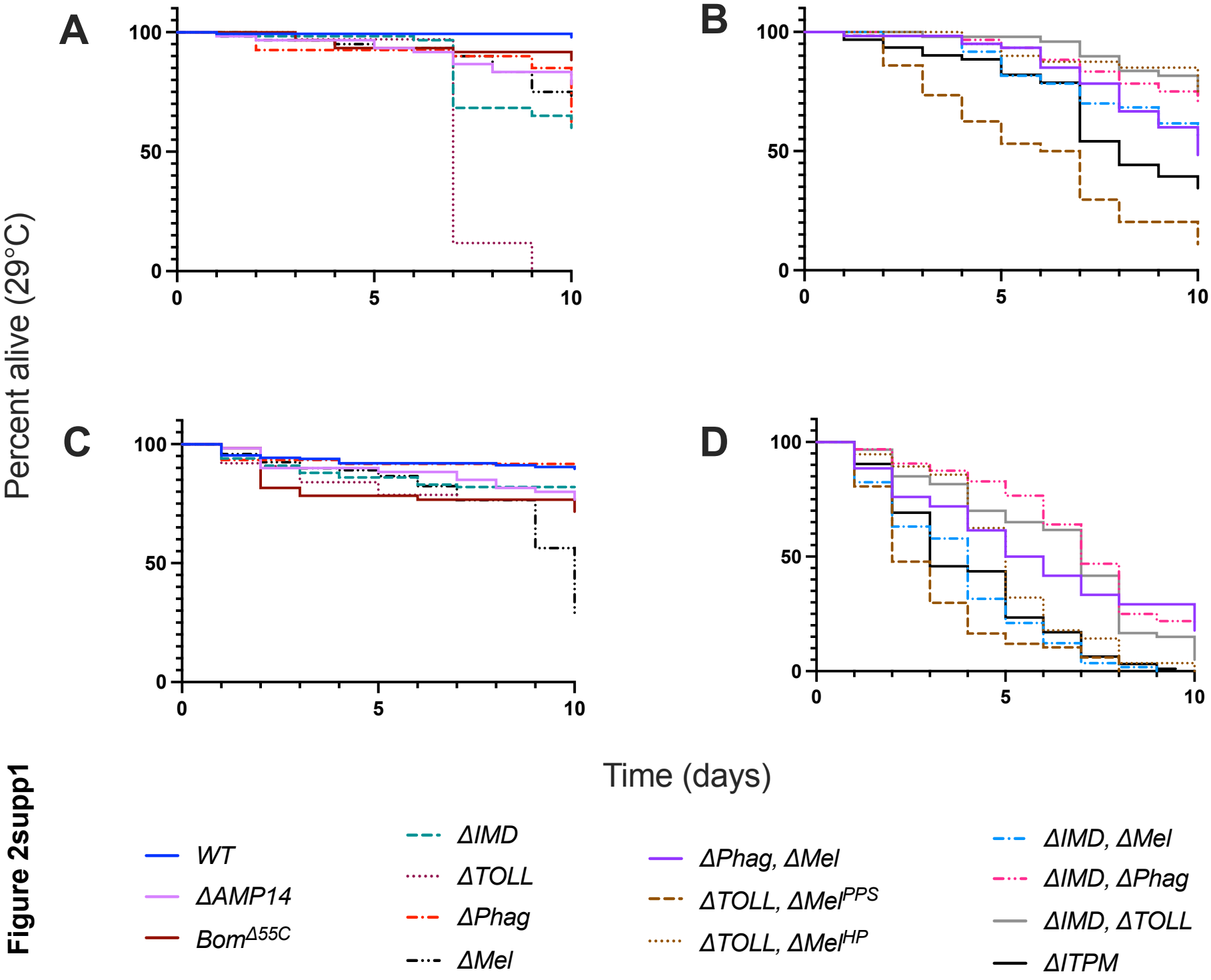
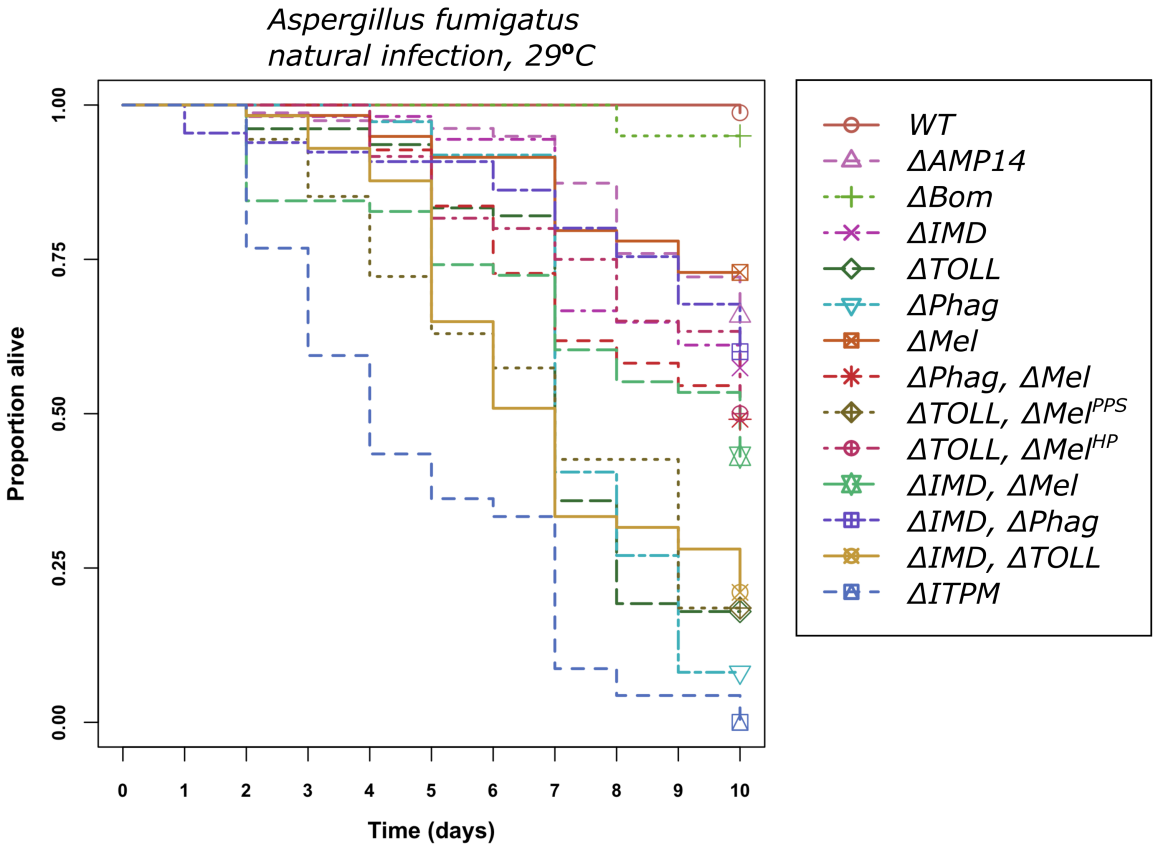


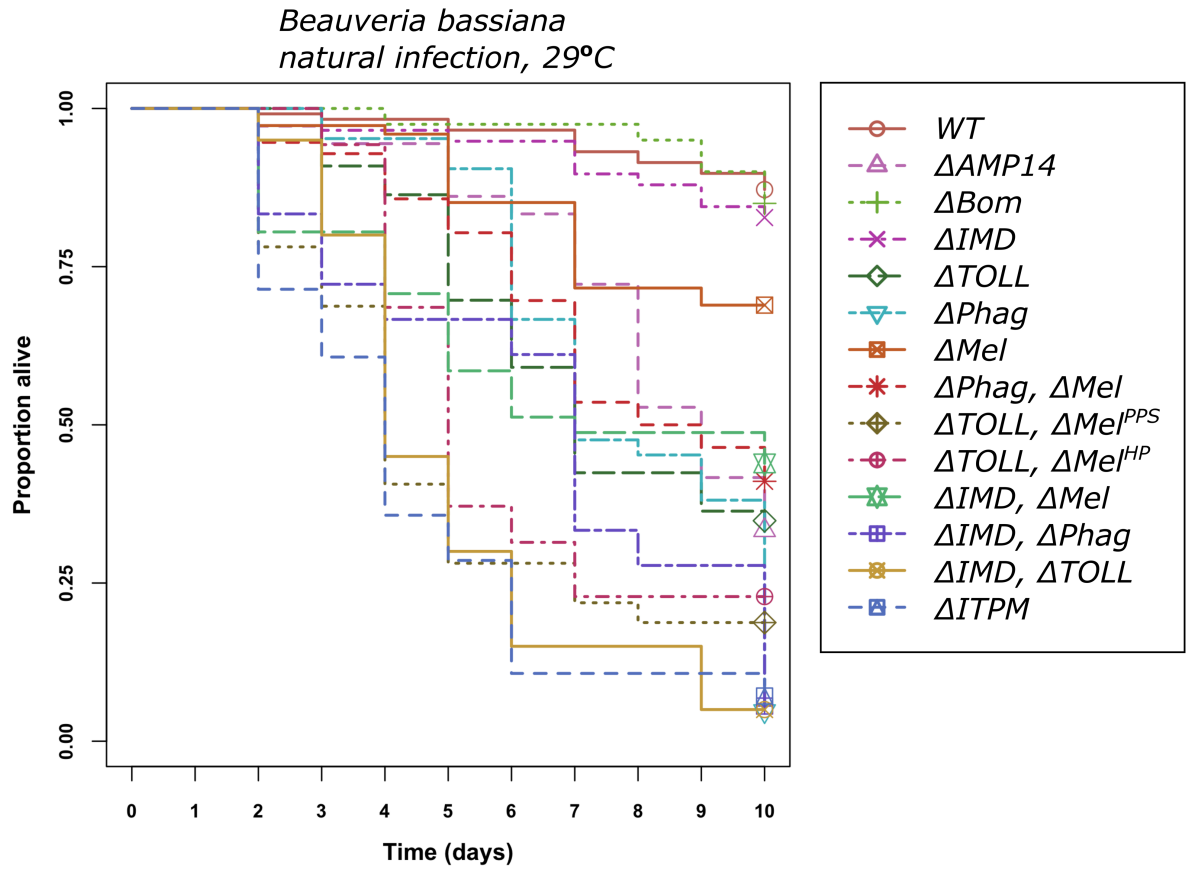
Figure 2supp1

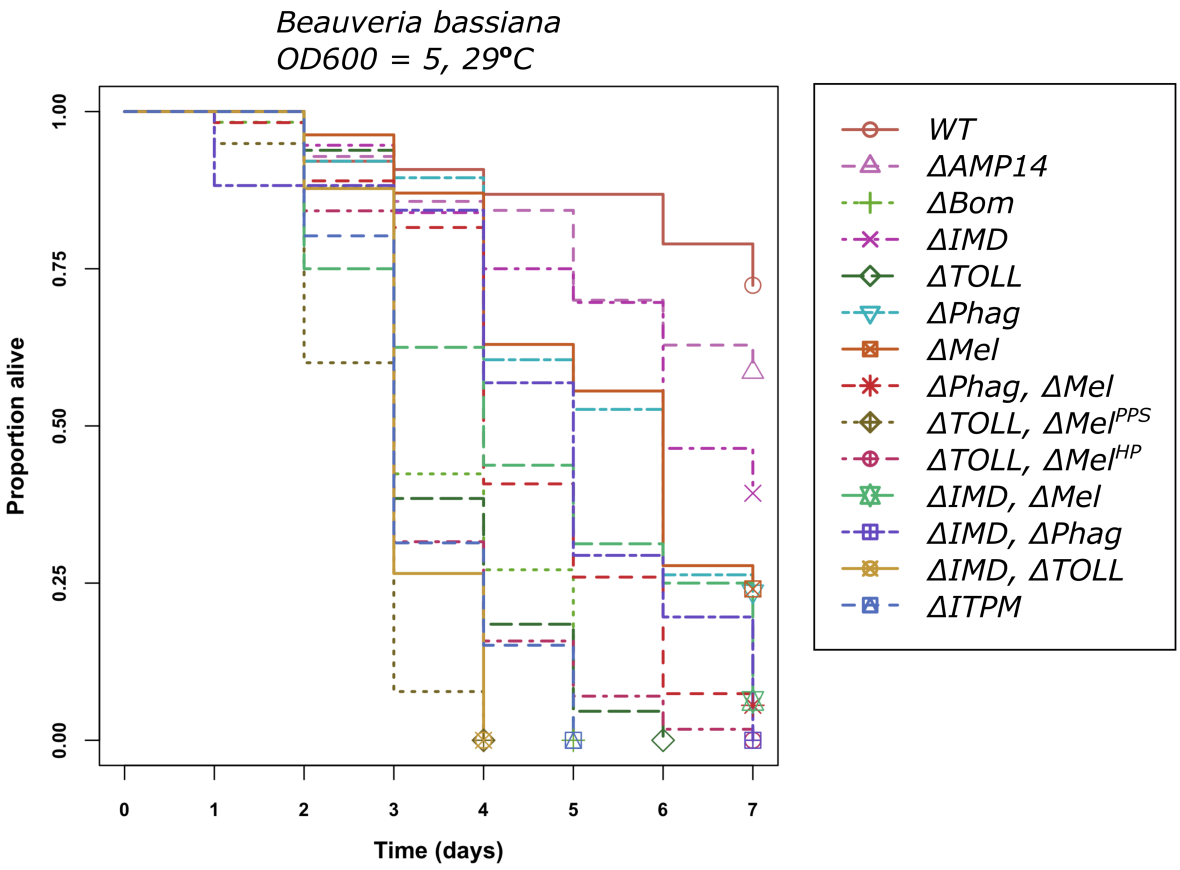
Figure 3supp1

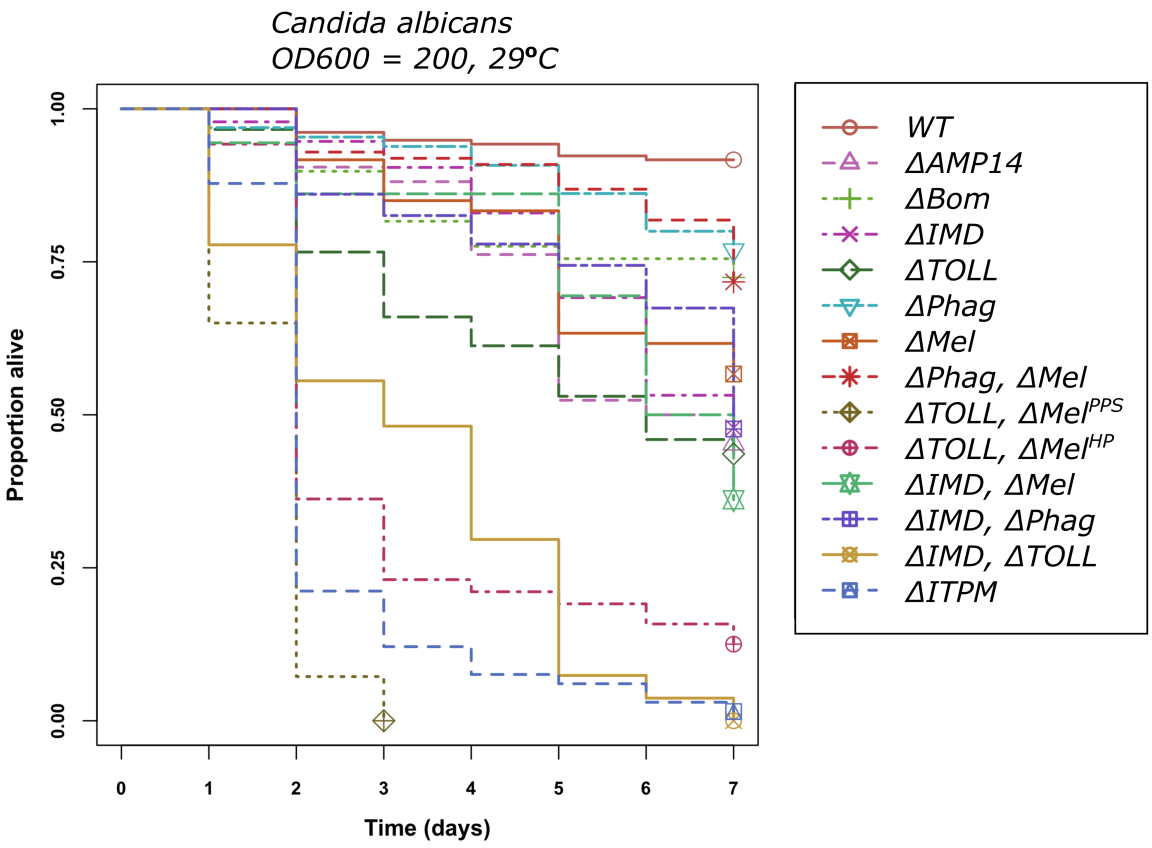
	Microbe	*AMP14*	*Bom*	IMD	TOLL	Phag	Mel
Viruses	DCV	✓		✓	(✓ I, M)		✓
	FHV	✓	✓	✓	✓	✓	✓
	DXV	✓		✓	✓	✓	✓
	IIV-6	✓		✓	(✓ I)		✓
	SINV			(✓ T, P)	(✓ I)	✓	✓
Fungi	*A. fumigatus (NI)*			(✓ T)	(✓ I, M)		(✓ T)
	B. bassiana (NI)	✓		(✓ P)	(✓ I, M)	(✓ I, M)	(✓ T, P)
	B. bassiana (SI)		✓		✓	✓	
	C. albicans (SI)	✓		(✓ T)	✓		(✓ T)
Gram positive bacteria	*M. marinum*						
	C. diphteriae			(✓ T)	(✓ I, M)		✓
	M. luteus			(✓ T)	(✓ I)		
	S. pneumoniae						
	E. faecalis		✓		✓	(✓ M)	(✓ P)
	S. aureus				✓	✓	✓
	L. monocytogenes		✓	✓	✓		
	B. subtilis	✓	✓	✓	✓		(✓ T)
Gram negative bacteria	*V. parahemolyticus*	✓		✓	✓		
	P. rettgeri	✓		✓	✓	✓	✓
	P. burhodogranaria	✓		✓	(✓ M)		✓
	Ecc15	✓	✓	✓		✓	
	K. pneumoniae	✓		✓			
	E. cloacae	✓		✓			
	E. coli	✓		✓	(✓ I)	✓	
	S. enterica typhimurium	✓		✓	(✓ I)	(✓ I)	

Supplementary file 1
Fungi

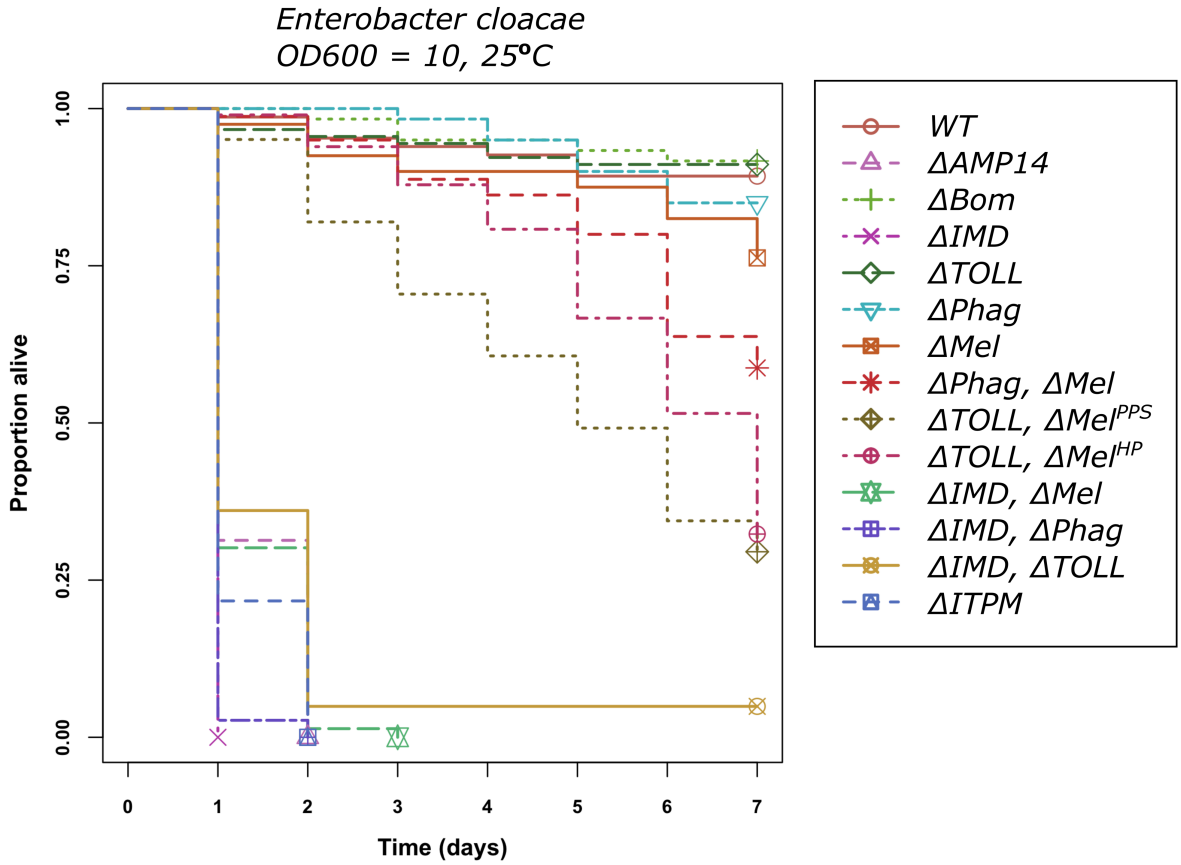


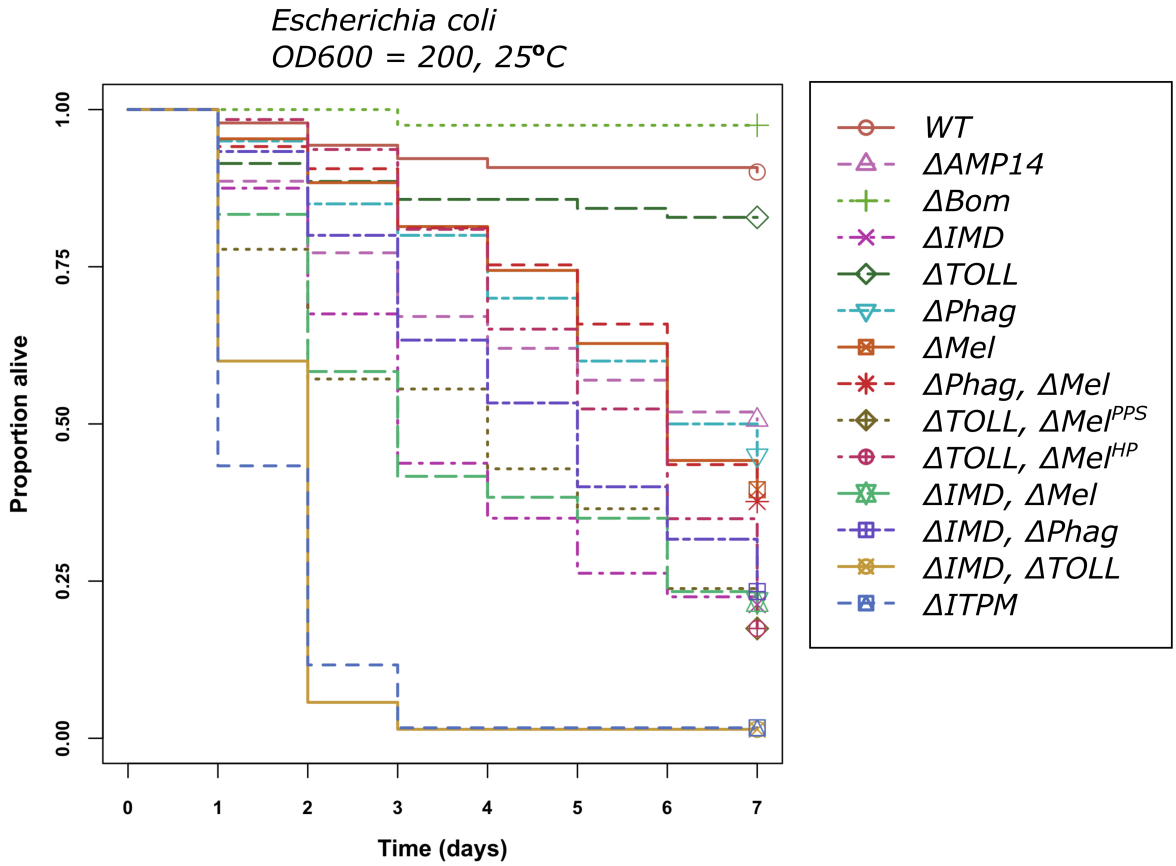


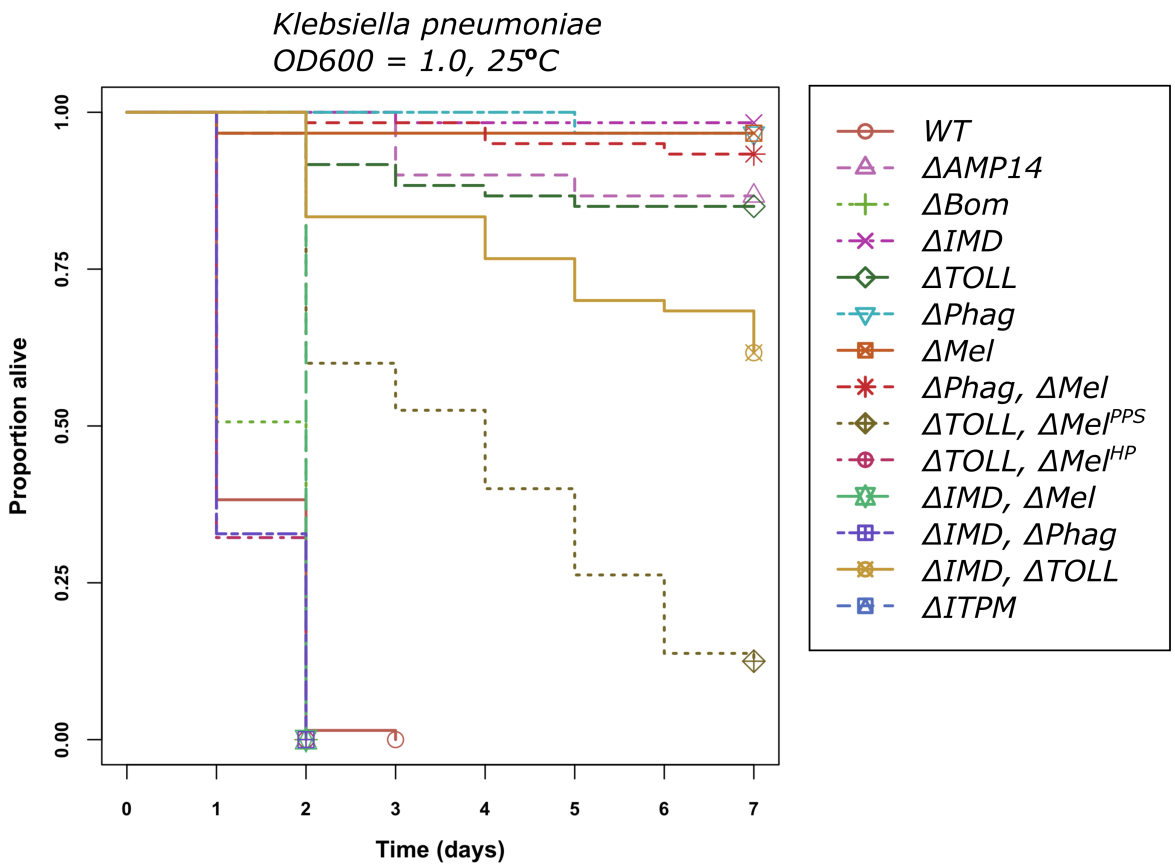

Beauveria bassiana
OD600 = 5, 29°C
Proportion alive
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM

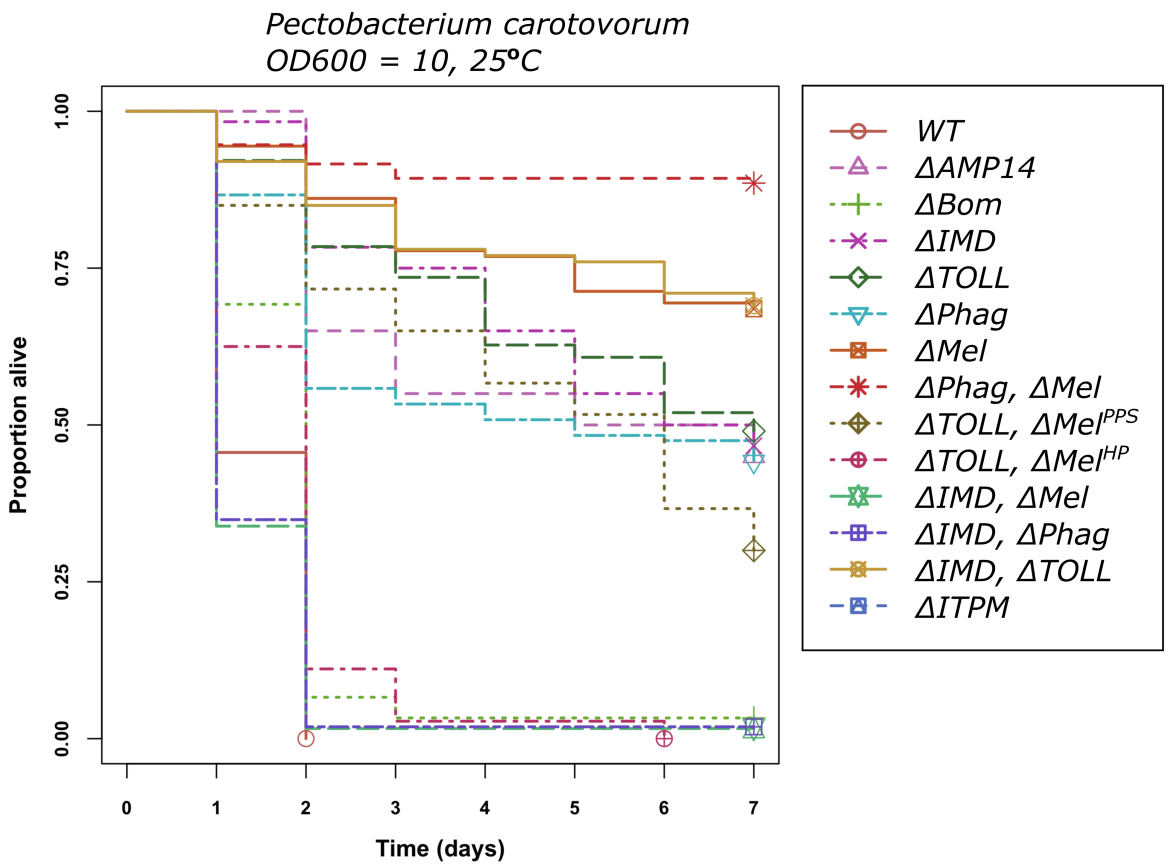

Candida albicans
OD600 = 200, 29°C
Proportion alive
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM

Gram-negative bacteria

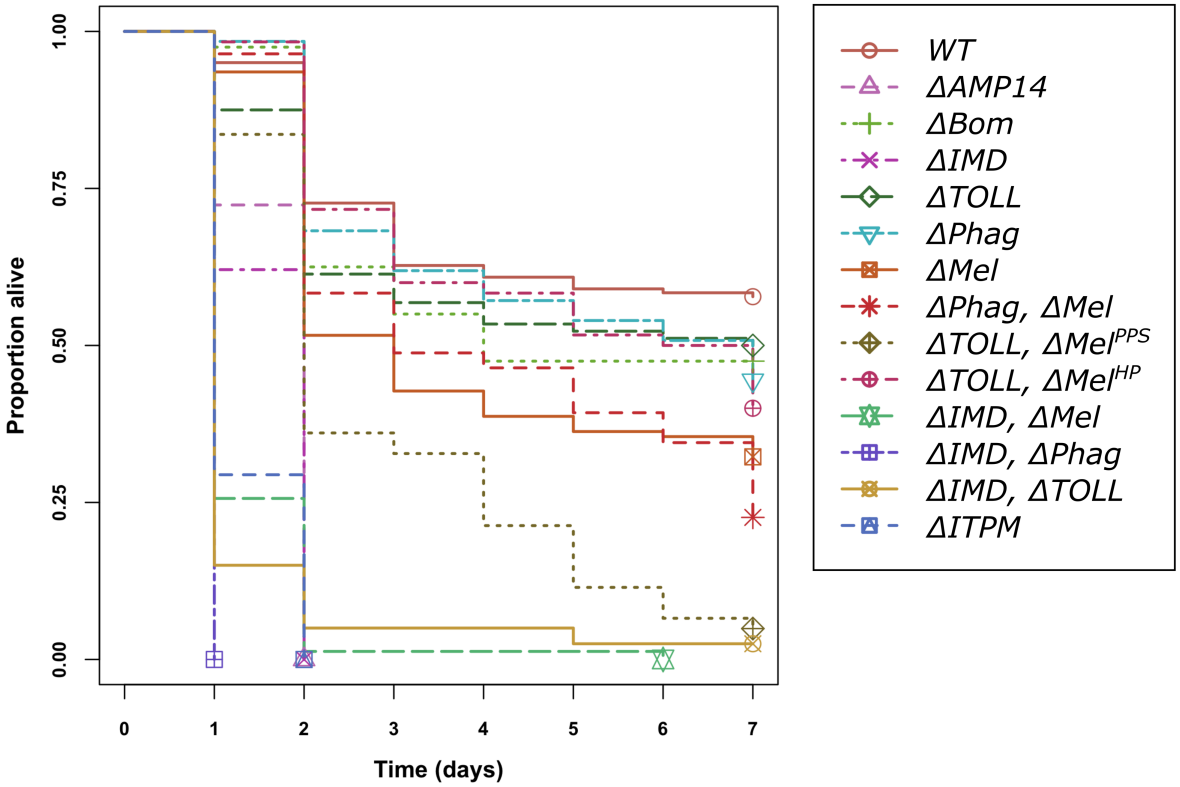




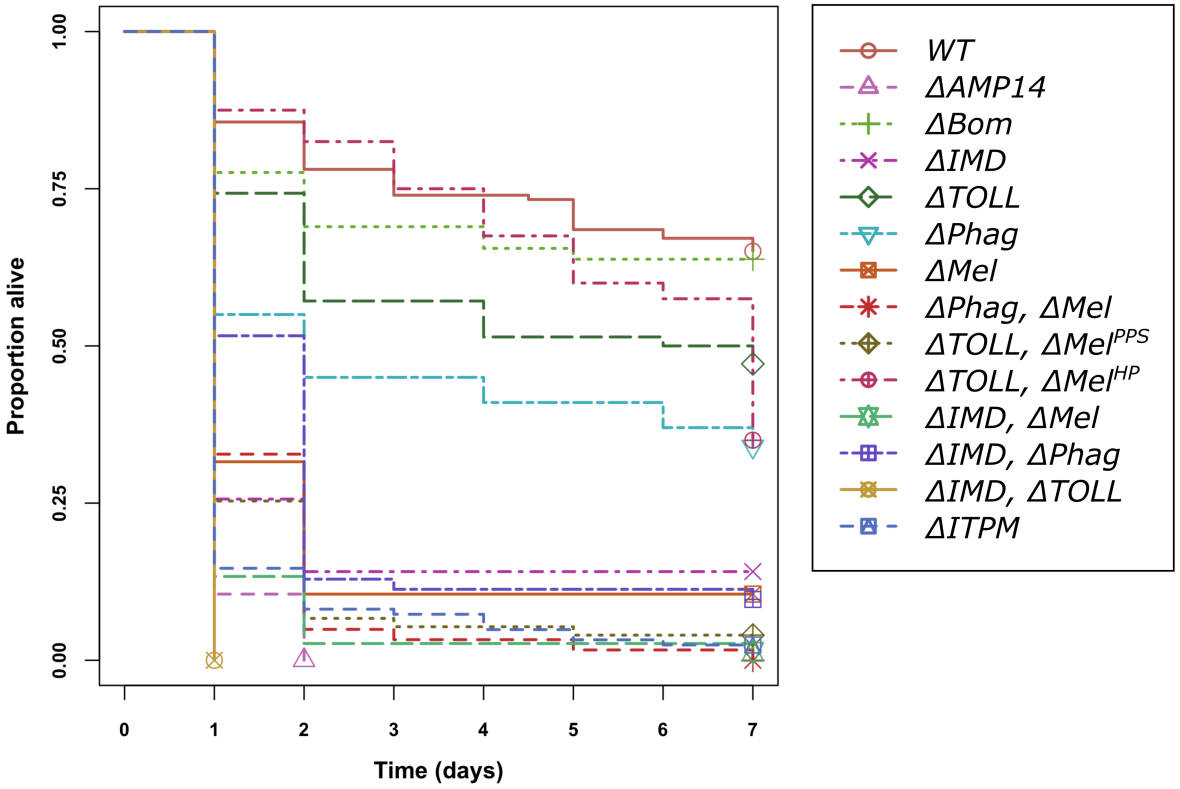

Klebsiella pneumoniae
OD600 = 1.0, 25°C
Proportion alive
0.00
0.25
0.50
0.75
1.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM

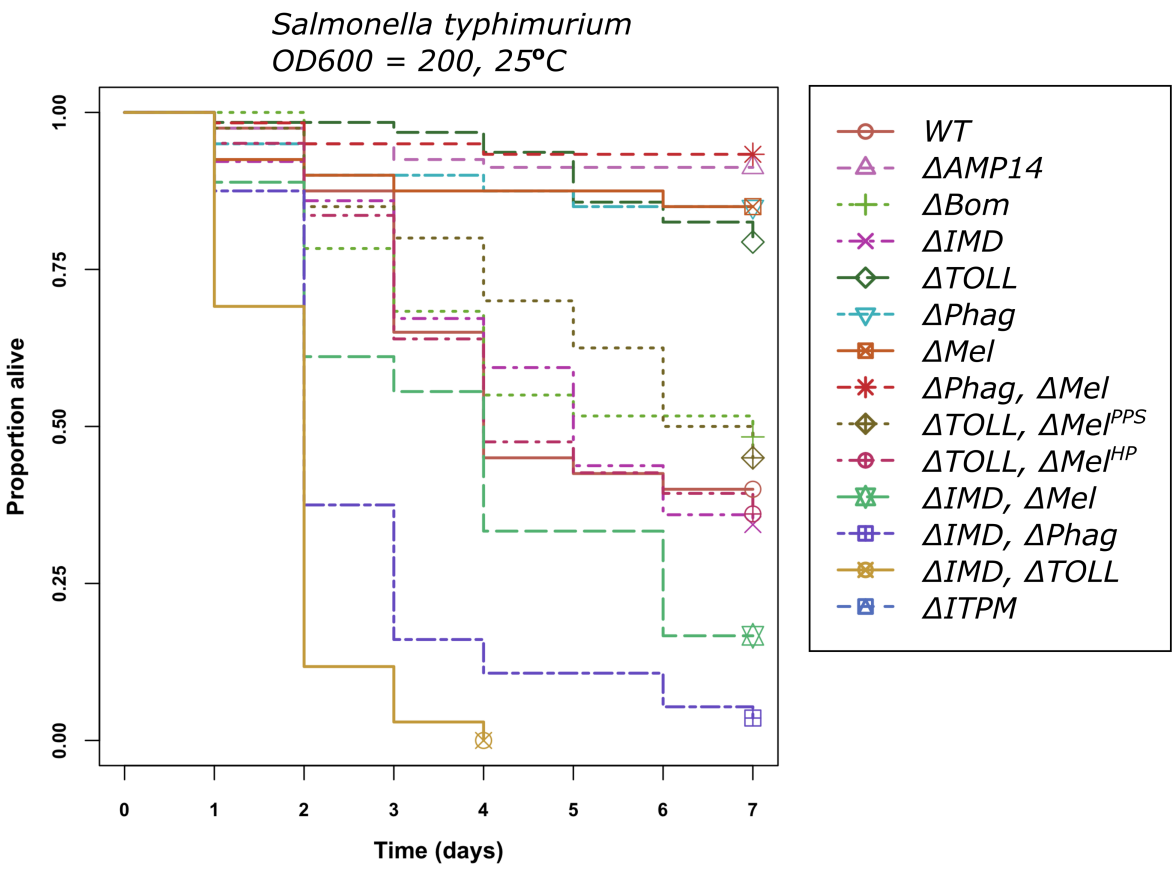

Pectobacterium carotovorum
OD600 = 10, 25°C
Proportion alive
0.00
0.25
0.50
0.75
1.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM

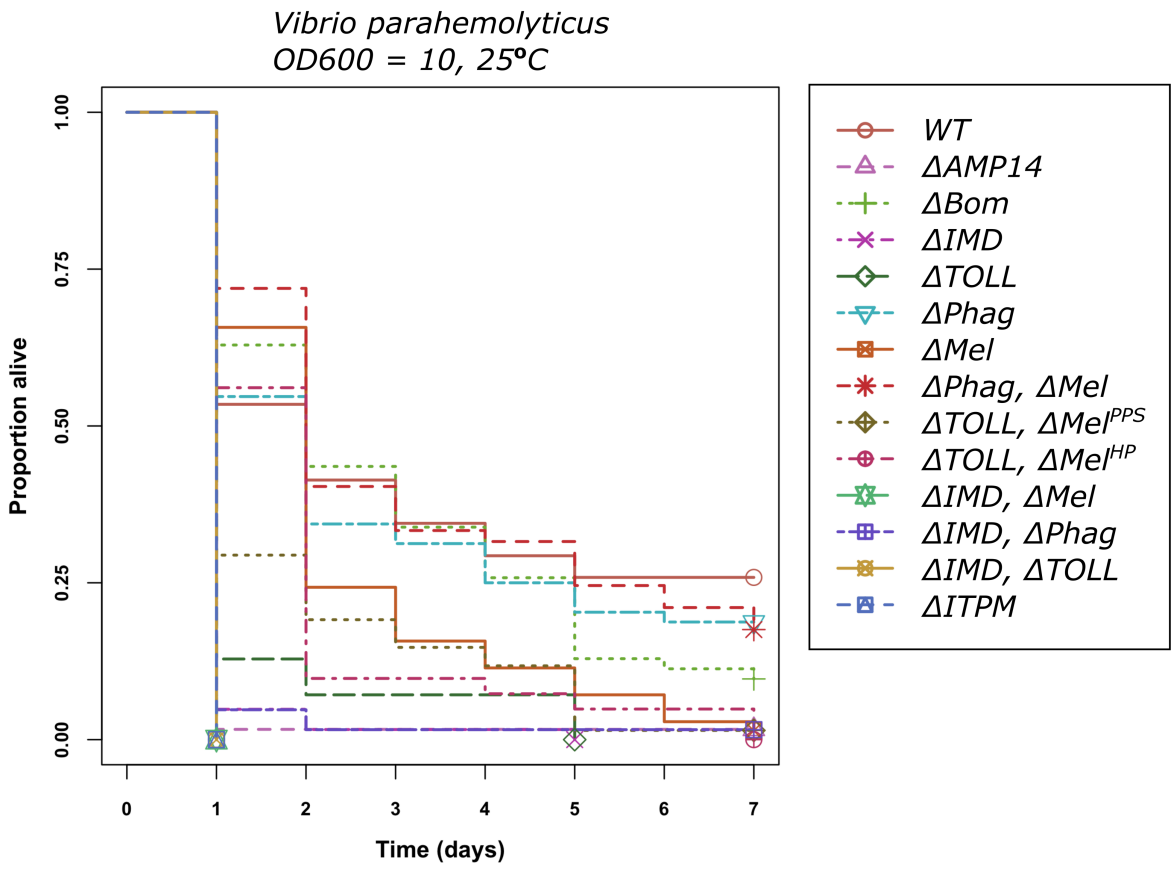
Providencia burhodogranariea
OD600 = 10, 25°C



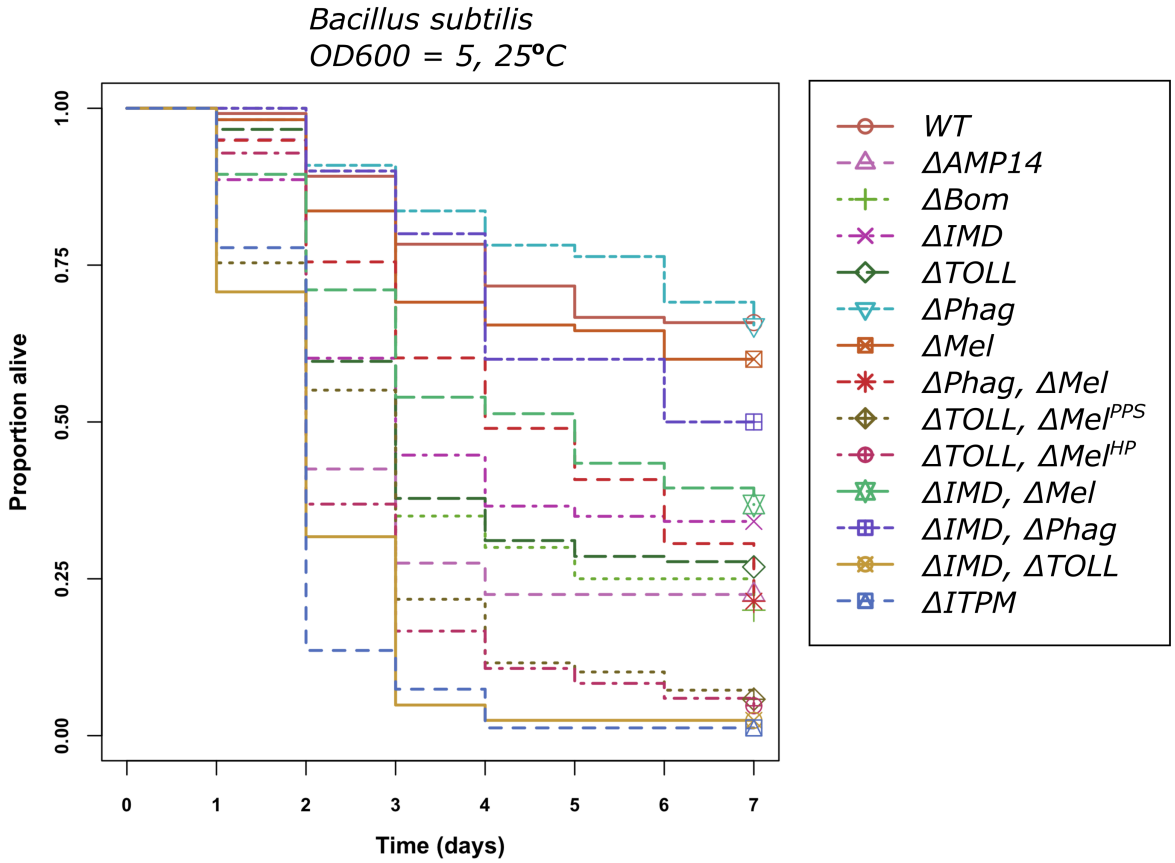
Providencia rettgeri
OD600 = 0.1, 25°C

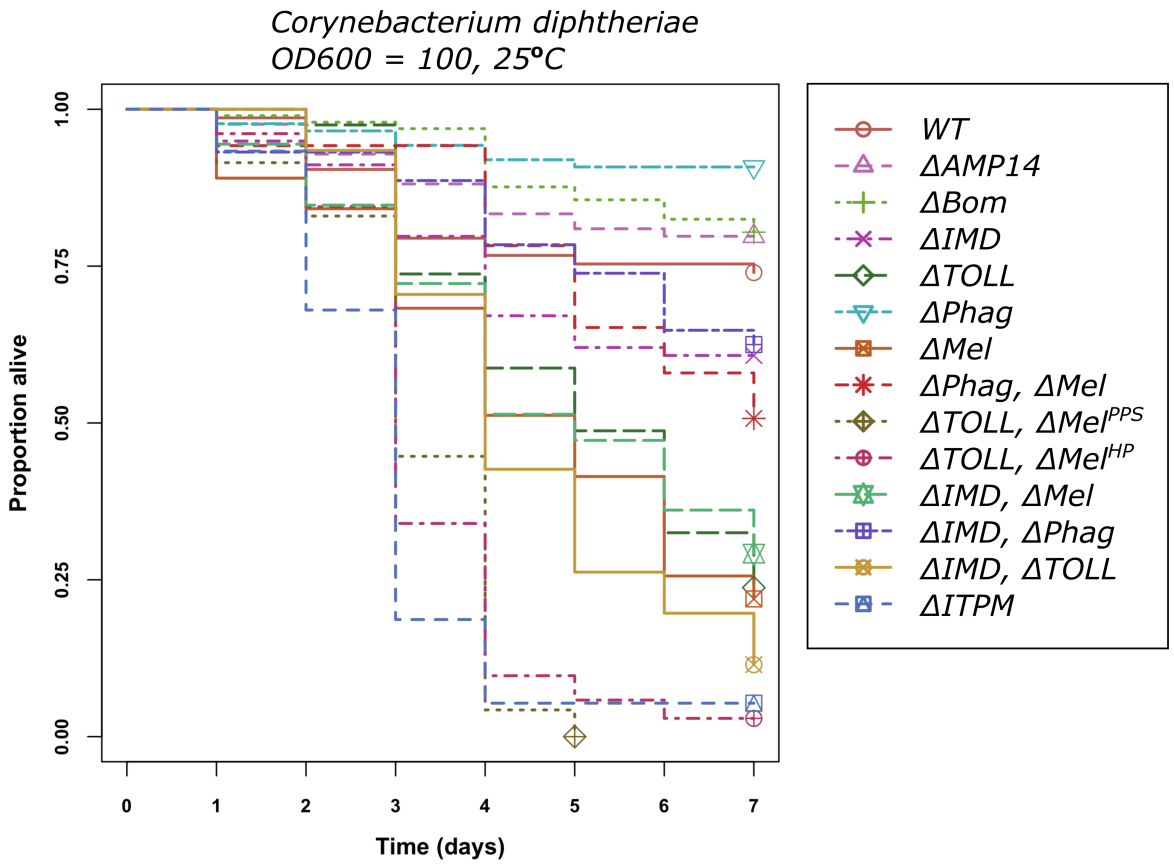


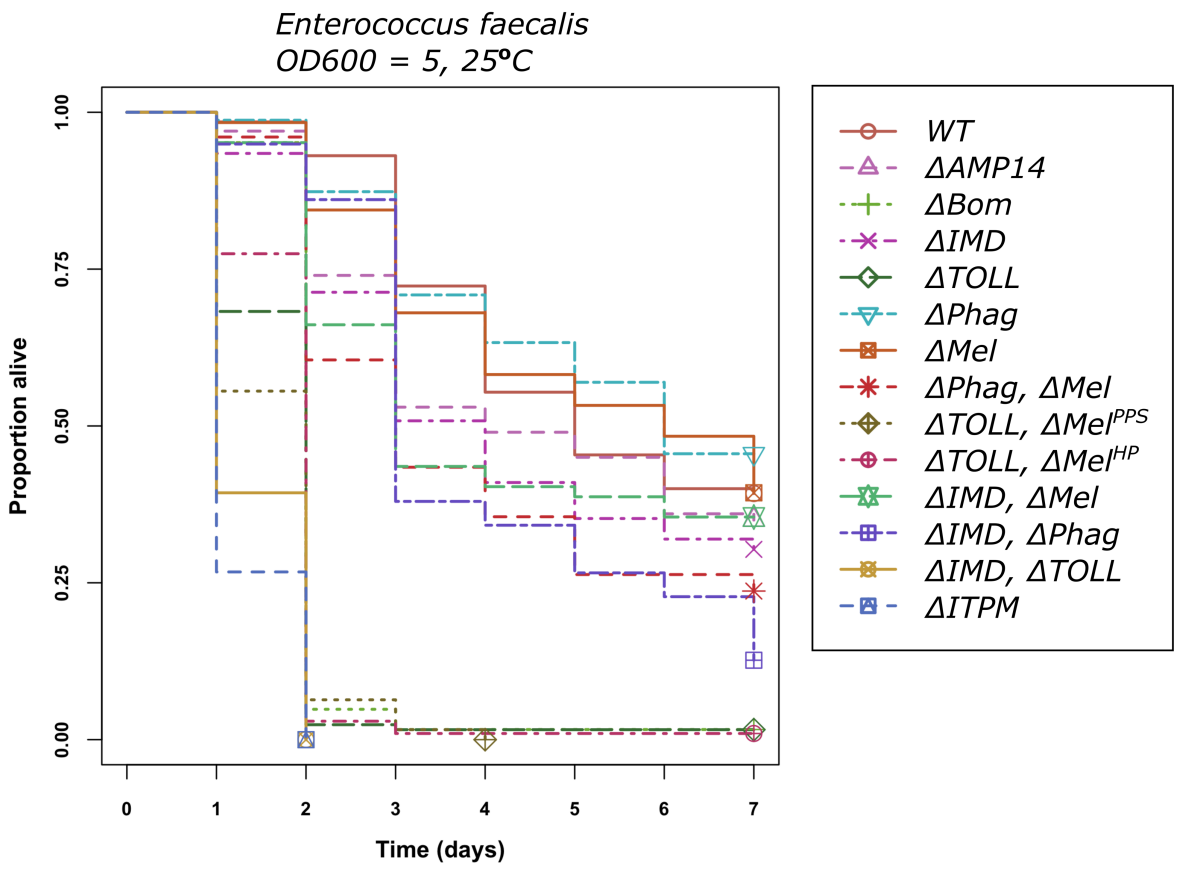

Salmonella typhimurium
OD600 = 200, 25°C
Proportion alive
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM

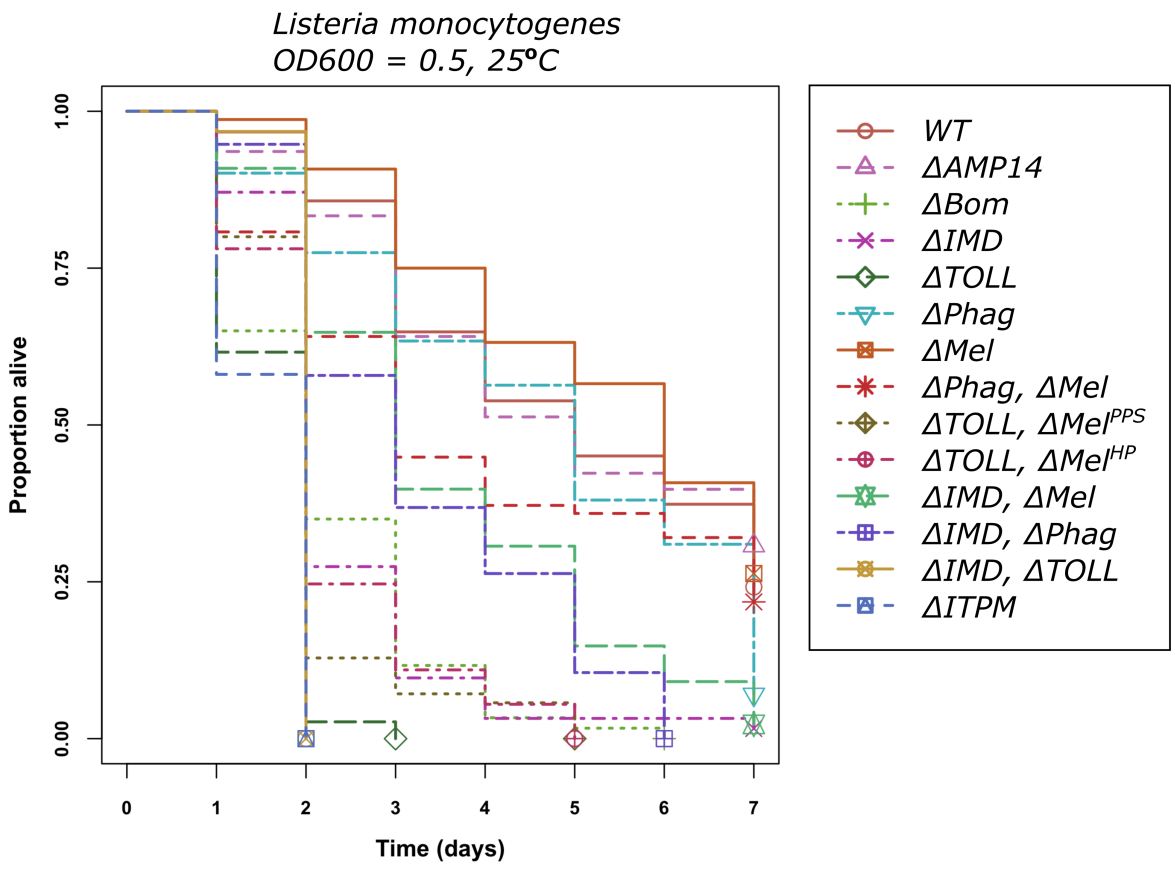

Vibrio parahemolyticus
OD600 = 10, 25°C
Proportion alive
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM

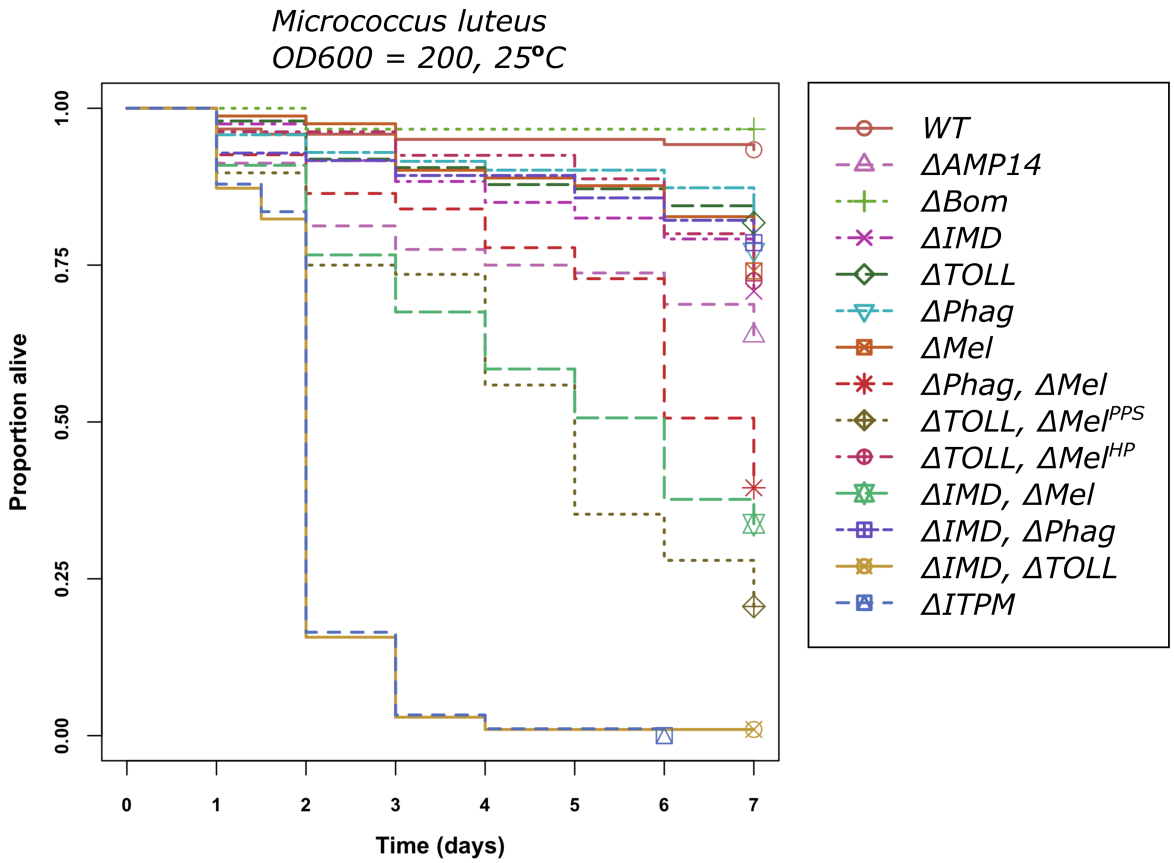
Gram-positive bacteria

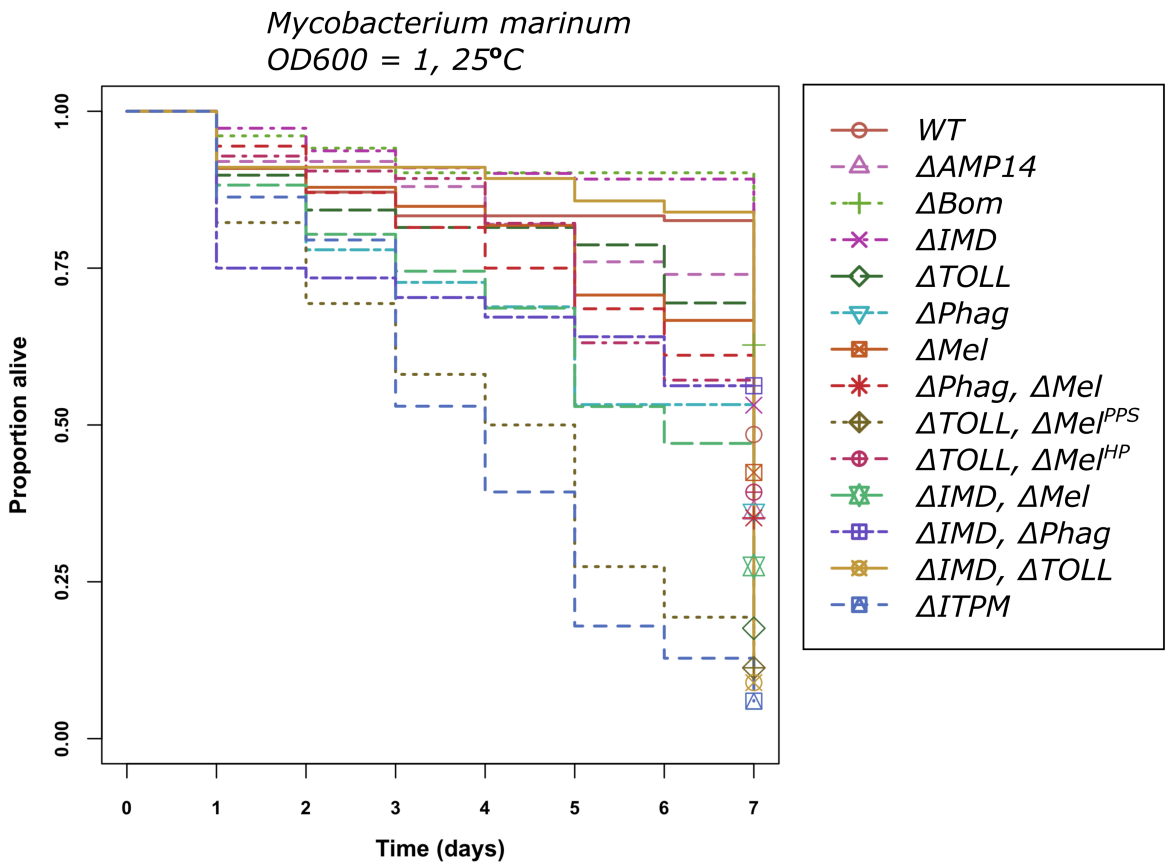


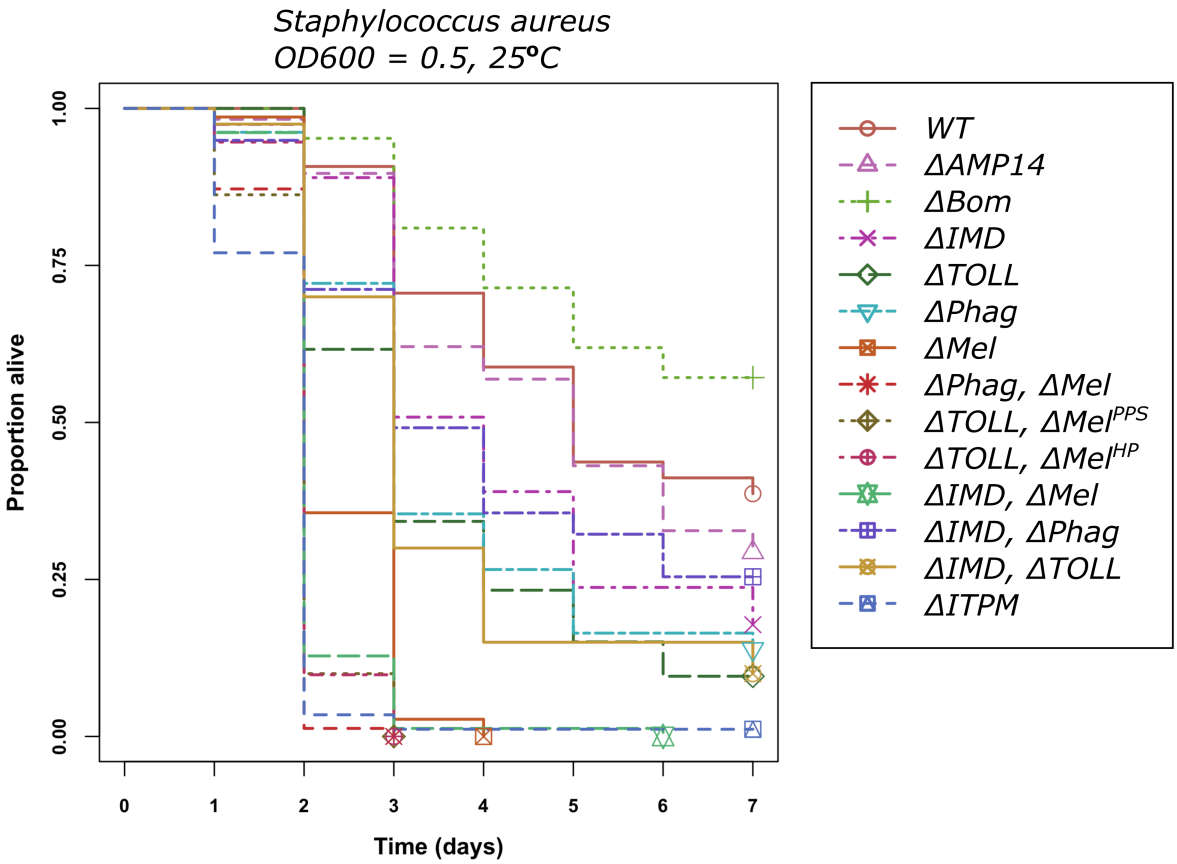


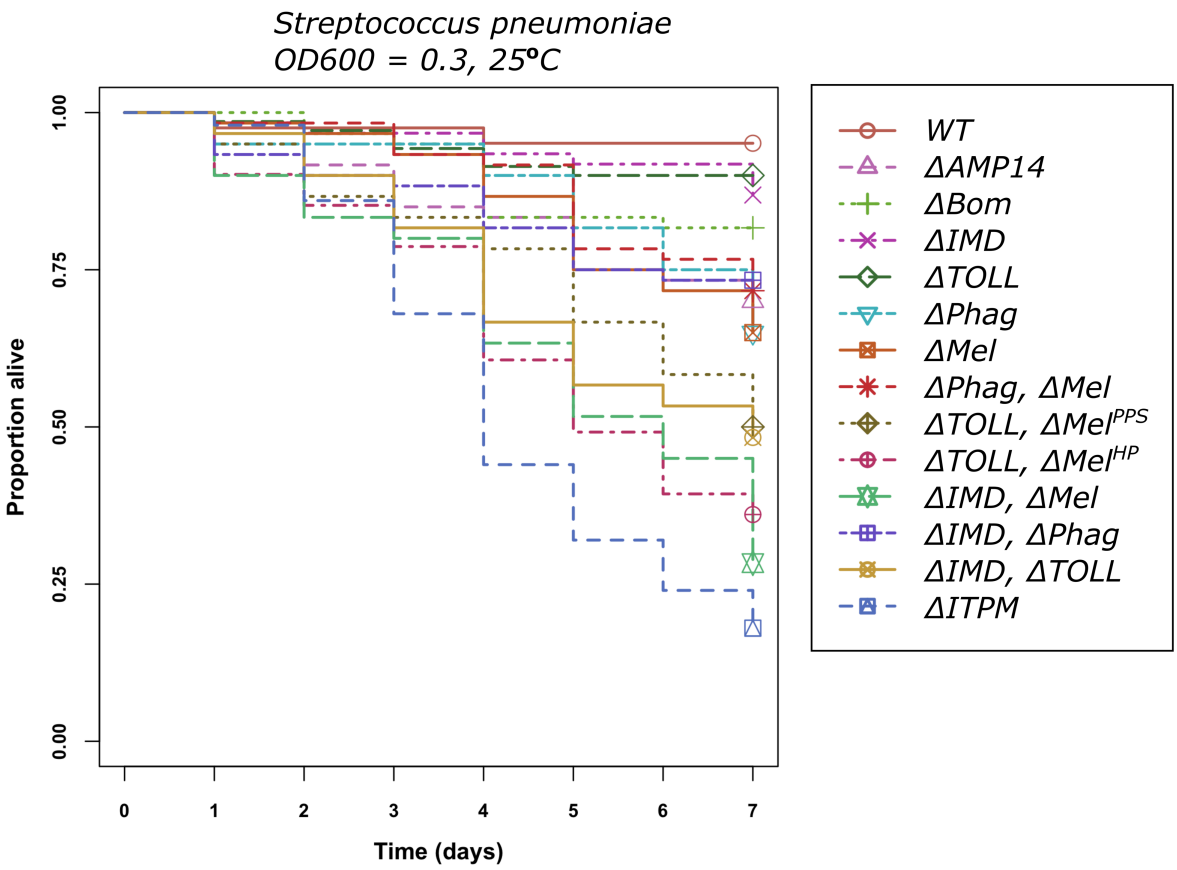

Enterococcus faecalis
OD600 = 5, 25°C
Proportion alive
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM


Listeria monocytogenes
OD600 = 0.5, 25°C
Proportion alive
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM


Micrococcus luteus
OD600 = 200, 25°C
Proportion alive
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM

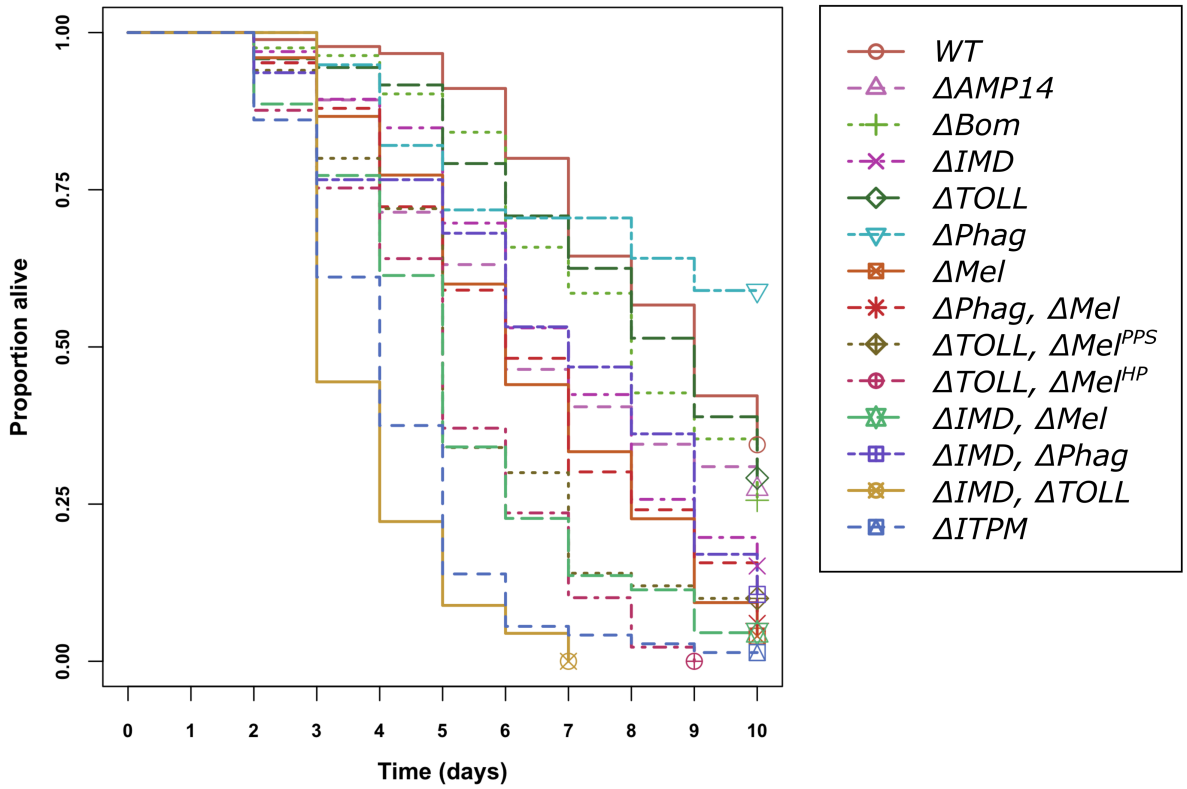

Mycobacterium marinum
OD600 = 1, 25°C
Proportion alive
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM


Staphylococcus aureus
OD600 = 0.5, 25°C
Proportion alive
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM

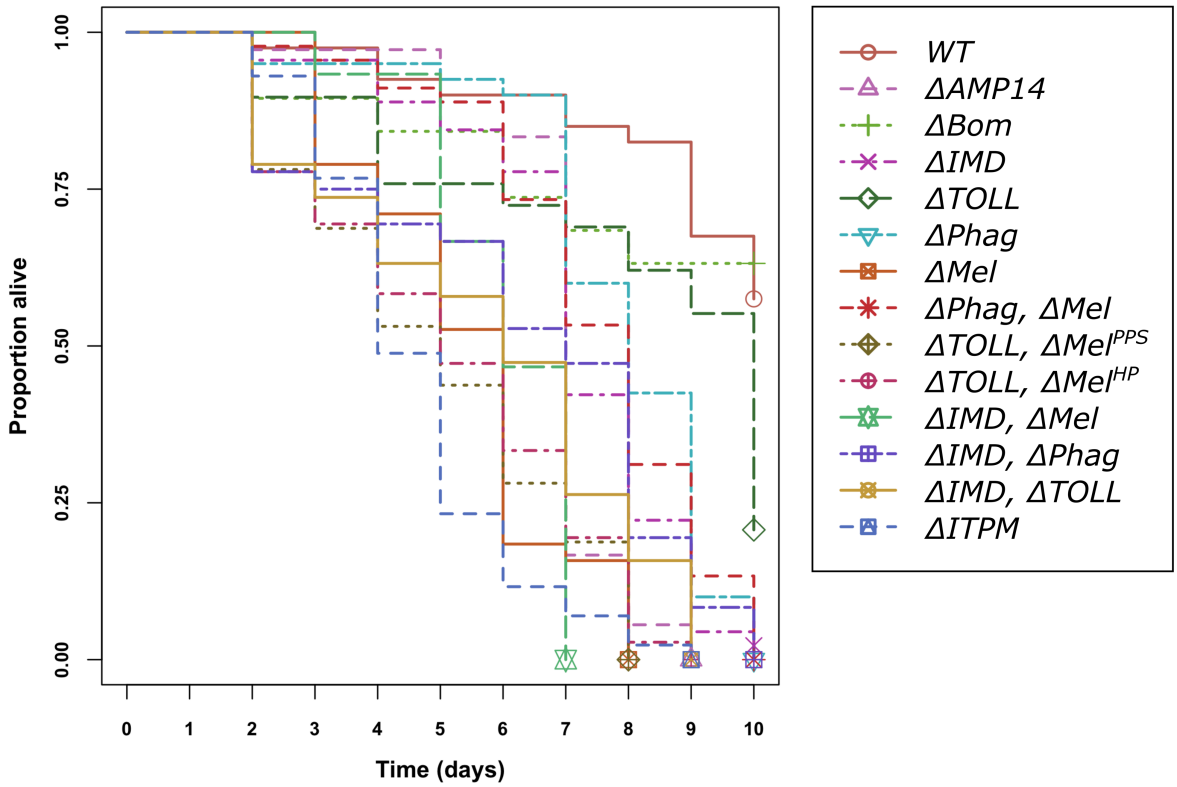

Streptococcus pneumoniae
OD600 = 0.3, 25°C
Proportion alive
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM

Viruses

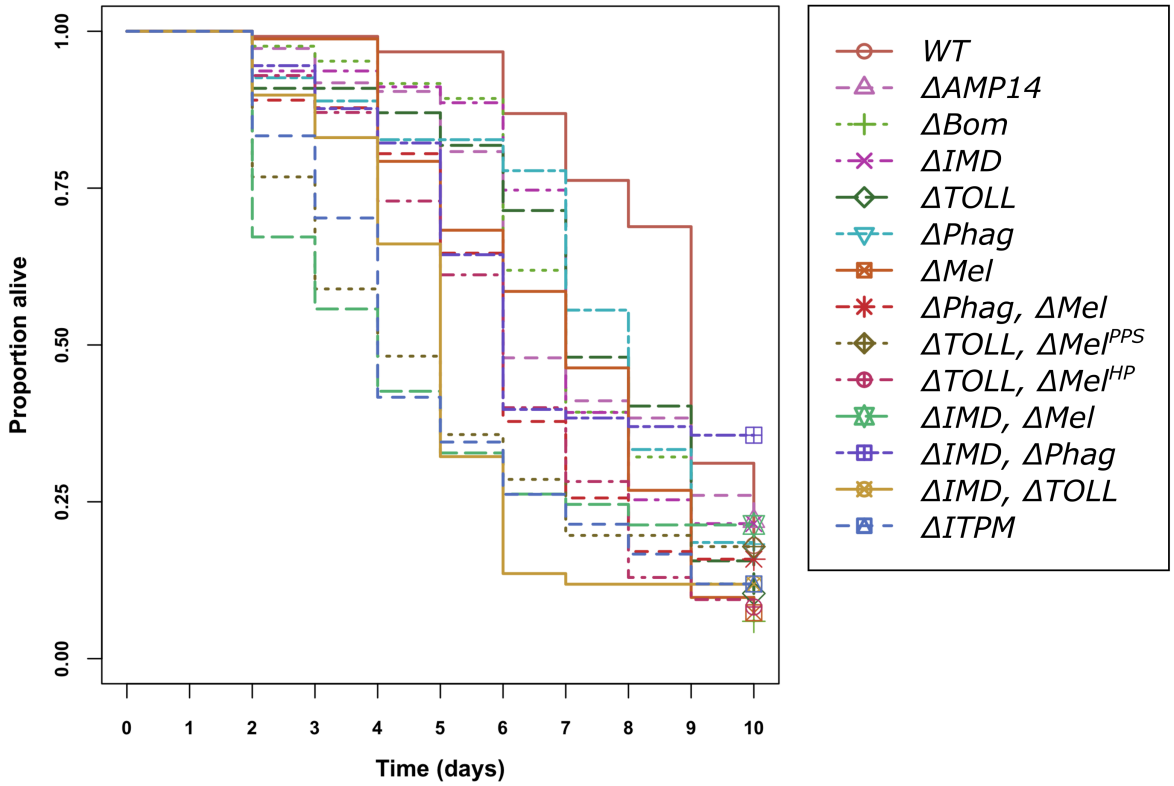
Drosophila C virus, 25°C



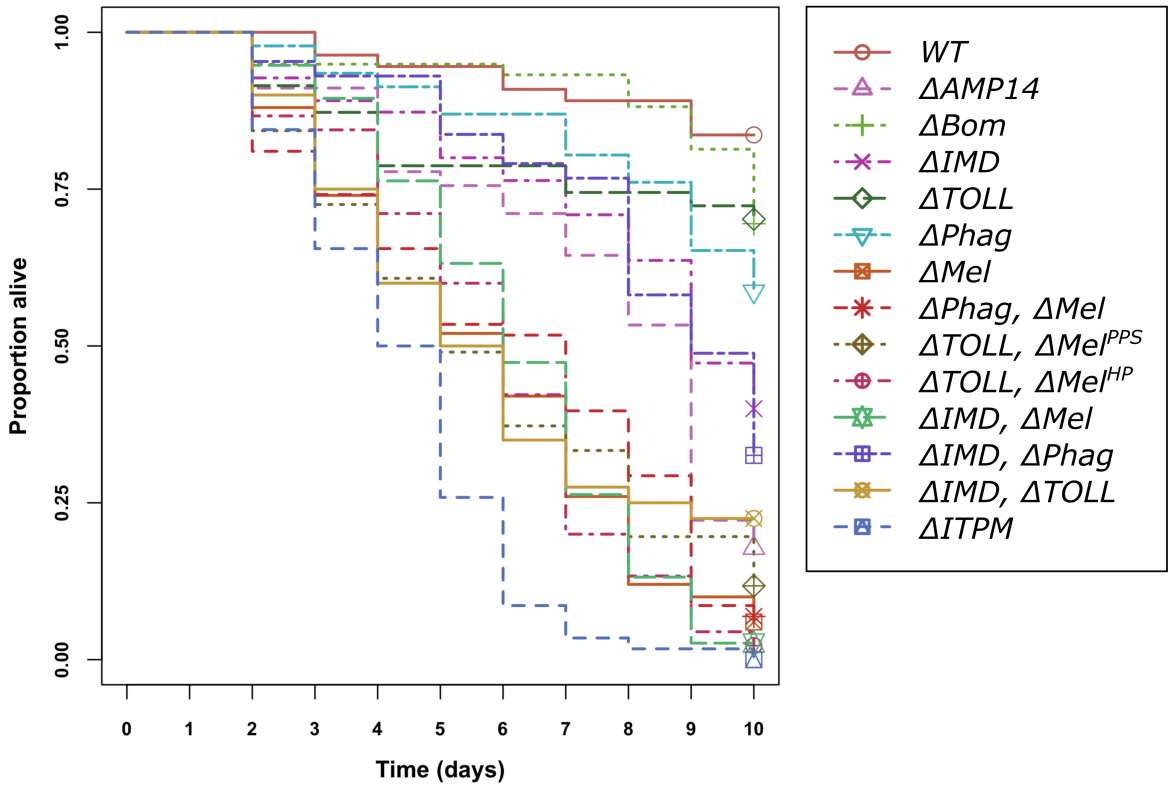
Drosophila X virus, 25°C

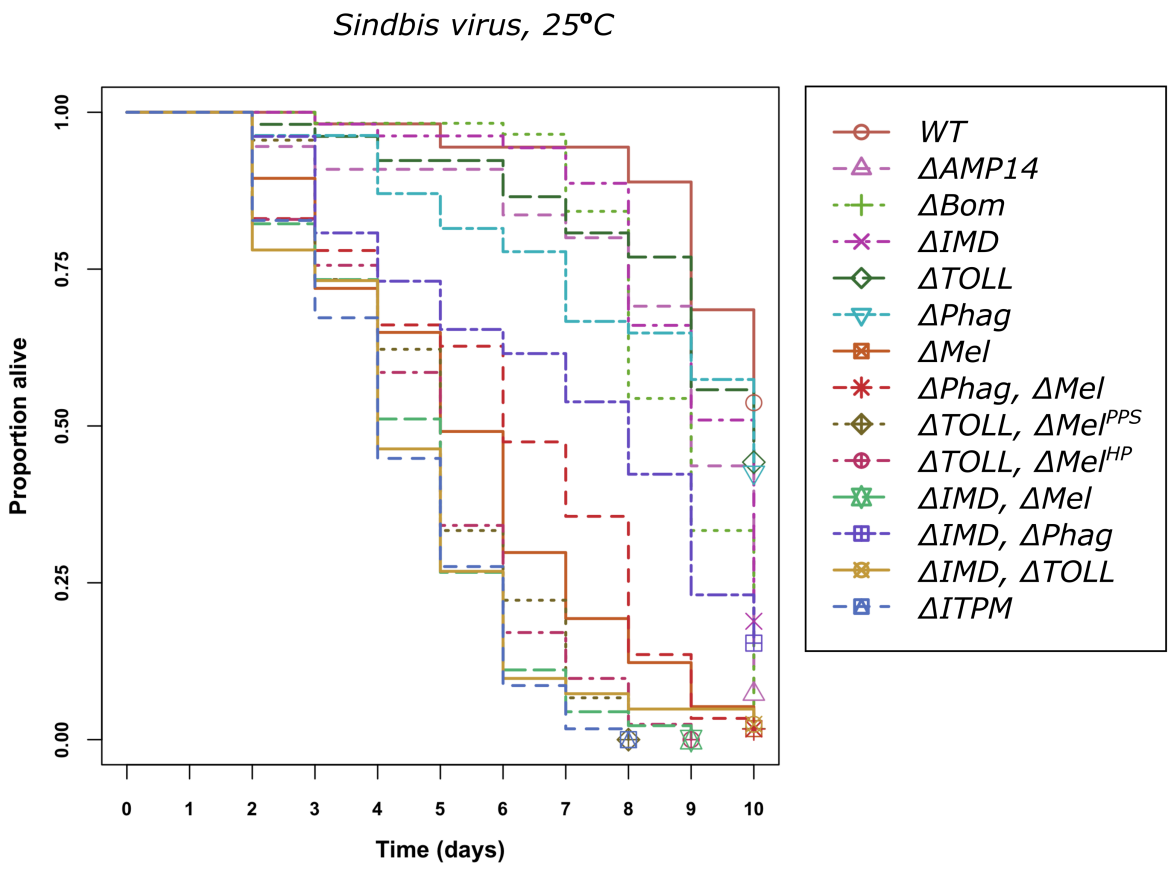


Flock house virus, 25°C



Invertebrate iridescent-6 virus, 25°C




Sindbis virus, 25°C
Proportion alive
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
8
9
10
Time (days)
WT
ΔAMP14
ΔBom
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM