Supporting Information Appendix for:

A Toll pathway effector protects Drosophila *specifically from distinct toxins secreted by a fungus or a bacterium*

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Movies S1 to S3
Tables S1-S4

Supplementary Material and Methods

Generation of CRISPR/Cas9-mediated null mutants

The *BaraA* (*CG18279*) and *CG18278* null mutants were generated using CRISPR/Cas9 technology based on the expression of gRNA transgenes that were then crossed to a transgenic line expressing a *pnos-Cas9* transgene. The 20bp-long gRNAs for the target genes were devised using web-based CRISPR Optimal Target Finder (http://targetfinder.flycrispr.neuro.brown.edu/). The plasmids carrying DNA sequences for the production of single strand gRNAs were constructed using standard methods. Briefly, the oligonucleotides were synthesized, denatured, and annealed to get double strand DNA before ligation into the expression vector, in which the gRNA coding sequences were transcribed under the control of the U6:3 promoter.

Plasmids carrying different gRNA targets were grouped by three or six for microinjection to obtain the gRNA transgenic fly lines, which were checked by sequencing. The gRNAs expressing plasmids were designed to be inserted on the 3rd chromosome using y^{1} M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP}ZH-86Fb (BL24749) flies. The gRNA flies were balanced before being crossed to flies carrying the *nosP-Cas9* transgene, to induce inheritable mutations. The primers used to generate the knock out mutants are shown in Table S3.

Knock-in strategy

PCRs were done with the Q5 Hot-start 2× master mix (New England BioLabs, NEB), and cloning was performed using the Gibson Assembly 2× Master Mix (NEB) following the manufacturer's instructions. The pCFD5 (U6:3-(t :: RNA^{Cas9})) plasmid vector was used. A cloning protocol to generate the pCFD5 plasmids encoding one to six tRNA-flanked sgRNAs was followed as described (1). The primers used to generate the pCFD5 vector containing the gRNAs are shown in Table S1. We used a pSK vector as donor plasmid with the homology arms flanking the mCherry:

a fragment 1552bp upstream of *BaraA* had been amplified as a left arm; a fragment 1952bp downstream of *CG30059* as a right arm. Left arm + mCherry + right arm have been assembled (Gilson Assembly) and the resulting fragment ligated to Pst1-Spe1 double-digested pSK and checked by sequencing. The plasmid mixture containing the two plasmids at a ration pCFD5:pSK=3:1, was injected into recipient y^1 M{Act5C-Cas9.P.RFP-}ZH-2A w^{1118} DNAlig4^{169} embryos.

Overexpression strategy

Normal PCRs in first and second round were performed to amplify the ORF of BaraA constructing in pDONR221 with attP site (2). The primers used are shown in Table S3. BP recombination reaction was performed using with DH5α competent cells (Invitrogen); next, sequence-confirmed ORF entry clones were transferred to the destination vector pGW-HA.attB using a Gateway LR reaction (Gibson assembly). After validation by sequencing, the plasmids were injected in a pool into y^1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP}ZH-86Fb embryos and missing constructs were reinjected alone.

Molecular mass fingerprints by MALDI MS

Each individual hemolymph sample was analyzed with the Bruker AutoFlex™ III based on Bruker Daltonics' smartbeam laser technology. The molecular mass fingerprints (MFP) were acquired using a sandwich sample preparation on a MALDI MTP 384 polished ground steel plate (Bruker Daltonics Inc., Germany). Briefly, the hemolymph samples were 10-fold diluted in acidified water (0.1% trifluoroacetic acid - 0.1% TFA, Sigma Aldrich, France), 0.6µL was deposited on a thin layer of an air-dried saturated solution (0.6µL) of the matrix

alpha-cyano-4-hydroxycinnamic Acid (4-HCCA, Sigma Aldrich, France) in pure acetone. Then 0.4 µL of a saturated solution of 4-HCCA prepared in 50% acetonitrile acidified with 0.1% TFA was mixed with the *Drosophila* hemolymph. Following co-crystallization of the hemolymph spots with the second matrix droplet and evaporation under mild vacuum, MALDI MS spectra were recorded in a linear positive mode and in an automatic data acquisition using FlexControl 4.0 software (Bruker Daltonics Inc.). The following instrument settings were used: the pseudo-molecular ions desorbed from the hemolymph were accelerated under 1.3kV, dynamic range of detection of 600 to 18,000 Da, between 50-60% of laser power, a global attenuator offset of 60% with 200Hz laser frequency, and 2,000 accumulated laser shots per hemolymph spectrum. The linear detector gain was setup at 1,906V with a suppression mass gate up to *m/z* 600 to prevent detector saturation by clusters of the 4-HCCA matrix. An external calibration of the mass spectrometer was performed using a standard mixture of peptides and proteins (Peptide Standard Calibration II and Protein Standard Calibration I, Bruker Daltonik) covering the dynamic range of analysis. All of the recorded spectra were processed with a baseline subtraction and spectral smoothing using FlexAnalysis 4.0 software (Bruker Daltonics Inc.).

Supplementary References

1. F. Port, S. L. Bullock, Augmenting CRISPR applications in Drosophila with tRNA-flanked sgRNAs. *Nat Methods* **13**, 852-854 (2016).
2. J. Bischof, E. M. Sheils, M. Bjorklund, K. Basler, Generation of a transgenic ORFeome library in Drosophila. *Nat Protoc* **9**, 1607-1620 (2014).

Legends to Supplementary movies

Supplementary movie 1

Wild-type (left) and *BaraA* KO1 (right) flies were inspected one hour after the injection of Destruxin A.

Supplementary movie 2

Wild-type (top) and *BaraA* KO1 (bottom) flies were inspected five hours after the injection of Destruxin A.

Supplementary movie 3

Wild-type (left) and *BaraA* KO1 (right) flies were inspected 22 hours after the injection of Destruxin A.

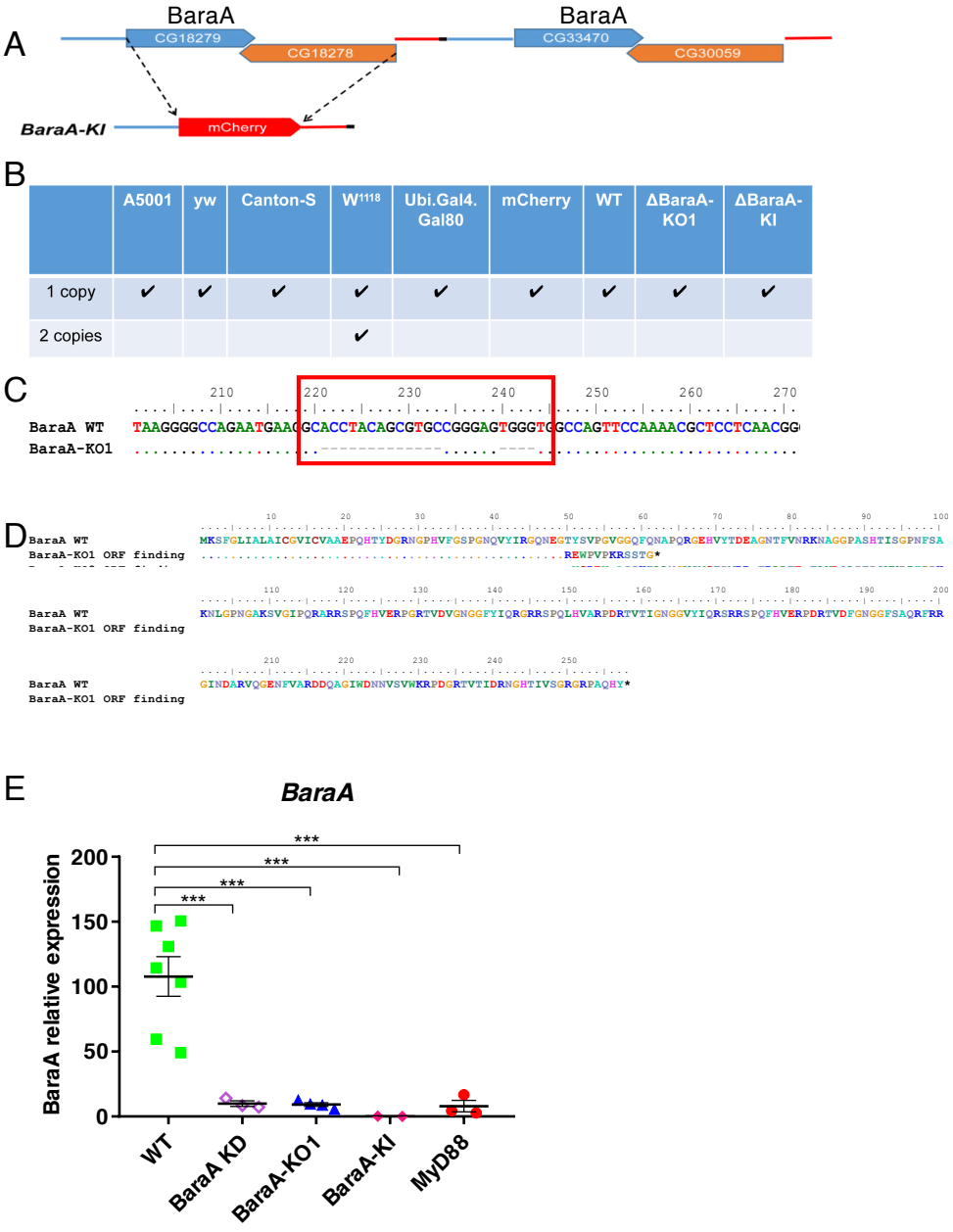

Figure S1. Mutants affecting the BaraA locus.

(A) Scheme of the tandem duplication of the *BaraA/CG18278(CG30059)* locus according to the Drosophila genome sequence; *CG18279* and *CG33470* (*BaraA*) on the one hand and *CG18278* and *CG30059* are perfectly duplicated, including 1172bp 5' to *CG18279* or *CG33470* start codon (shown as a blue line) and 774bp 5' to *CG18278* or *CG30059* start codon (shown as a red line). The black line represents the short unique region at the overlap of the duplicated loci. In the KI fly line, the two genes (*CG18279*, *CG18278*) were replaced by *mCherry* coding sequence after the START codon from *CG18279*. **(B)** Table recapitulating the tested strains and the presence of the duplication. The KI line was originally generated in a *yw* background with only one copy of the locus. **(C)** CRISPR Cas9 knock out mutants of *BaraA*: KO1 has a complex deletion pattern removing 17bp in total. **(D)** The small deletions found in the KO1 line leads to a frame shift mutation that generates an early stop codon (*). **(E)** *BaraA* expression level measured by RTqPCR in wild-type, knock down (KI), knock out (KO), knock in (KI), and *MyD88* flies, 24h after a *M. luteus* challenge. Data are expressed as means ± SEM. Pooled data from two independent experiments, **** $p<0.0001$

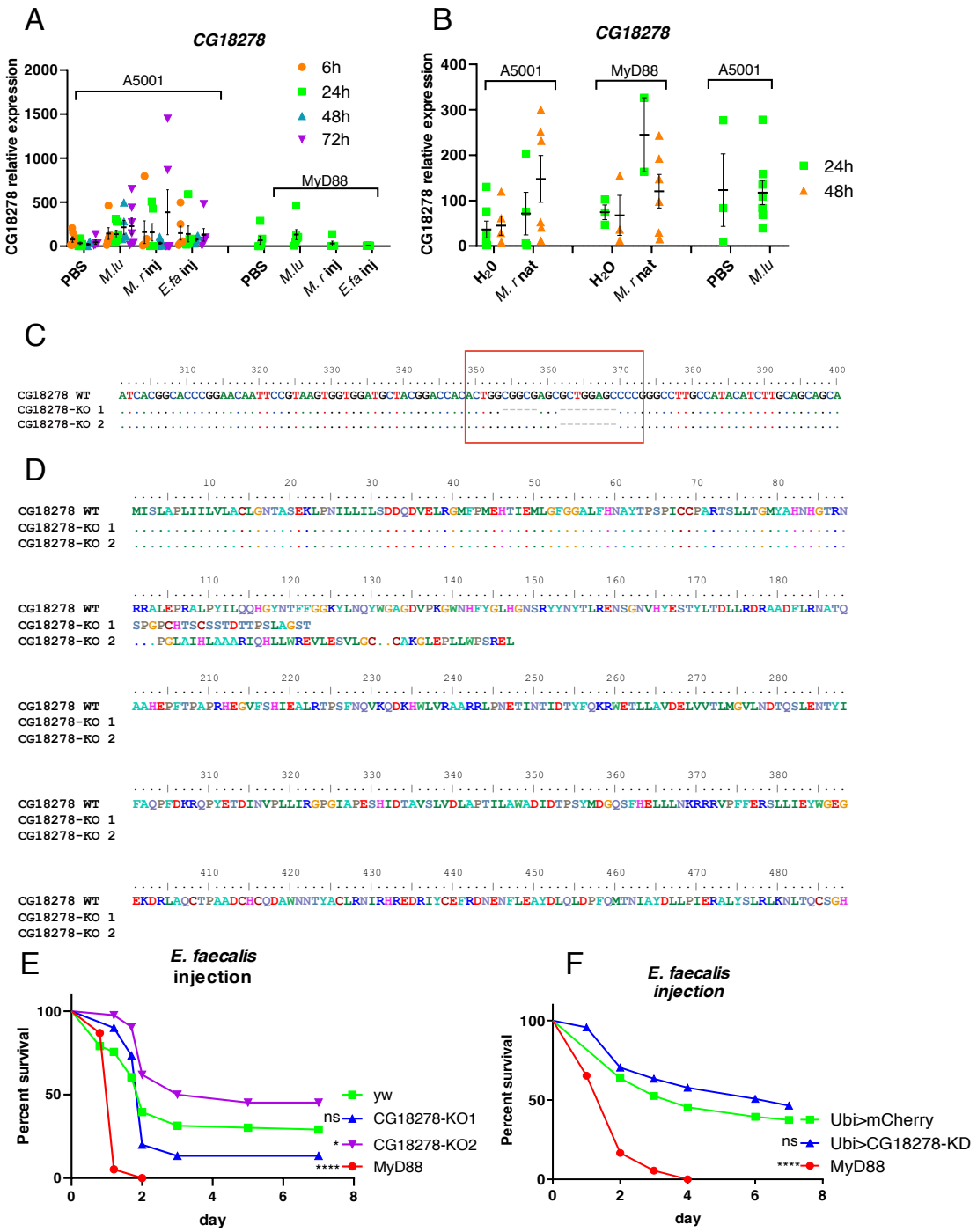

Figure S2. The *BaraA* neighboring gene *CG18278* is not involved in host defenses against *E. faecalis*.

(A) Expression of the *CG18278* gene monitored by RTqPCR in wild-type w^{A5001} and *MyD88* mutant flies at various time points after the injection of the indicated microbes; *M. lu: M. luteus*; *M. r: M. robertsii*; *E. fa: E. faecalis*. Data are expressed as means ± SEM. **(B)** Expression of the *CG18278* gene monitored by RTqPCR at 24 and 48 hours after a "natural" *M. robertsii* infection achieved by plunging the flies in a solution of conidia. Data were normalized to w^{A5001} with *M. luteus* challenged after 24 hours. Ct values from the RTqPCR for *CG18278* were in the 35-38 range while the Ct value for the Rpl32 were in the 18-20 range, which indicates that *CG18278* has low basal expression in fly. Data are expressed as means ± SEM. **(C, D)** Two lines of the CRISPR Cas9 mutant have been generated: *CG18278*-KO1 has a set of two small deletions removing altogether 13bp deletion whereas 8bp are deleted in the *CG18278* KO2 line. These deletions lead to frame shift mutations and early stop codons (D). **(E)** Nonisogenized *CG18278* KO1 mutant behaved like the *yw* reference line when infected by *E. faecalis* NCTC 775 while *CG18278* KO2 mutant displayed a slight protection compared to *yw*. Two independent experiments have been pooled, * $p<0.05$, **** $p<0.0001$. **(F)** Flies in which *CG18278* is attenuated by RNAi KD driven by *Ubi*-Gal4 displayed a sensitivity to *E. faecalis* NCTC 775 similar to that of the wild type. Two independent experiments have been pooled.

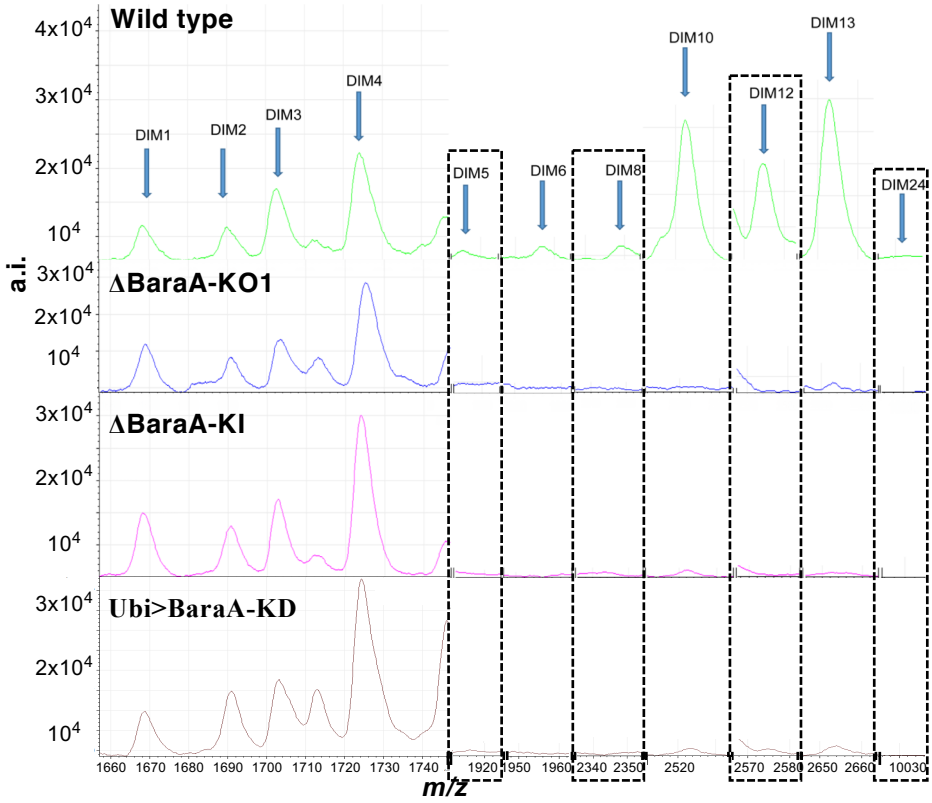

Figure S3. Detection of *BaraA*-derived DIM peptides in wild-type and *BaraA* KD, KO, and KI mutants by mass-spectrometry analysis.

The hemolymph was collected from single flies; four single flies were analyzed per genotype and yielded similar spectra by MALDI-TOF mass spectrometry. *BaraA* derived peptides were induced in wild type fly by *E. faecalis* NCTC 775. See also Table S1 for quantification of the peaks. a.i. : absolute quantitation.

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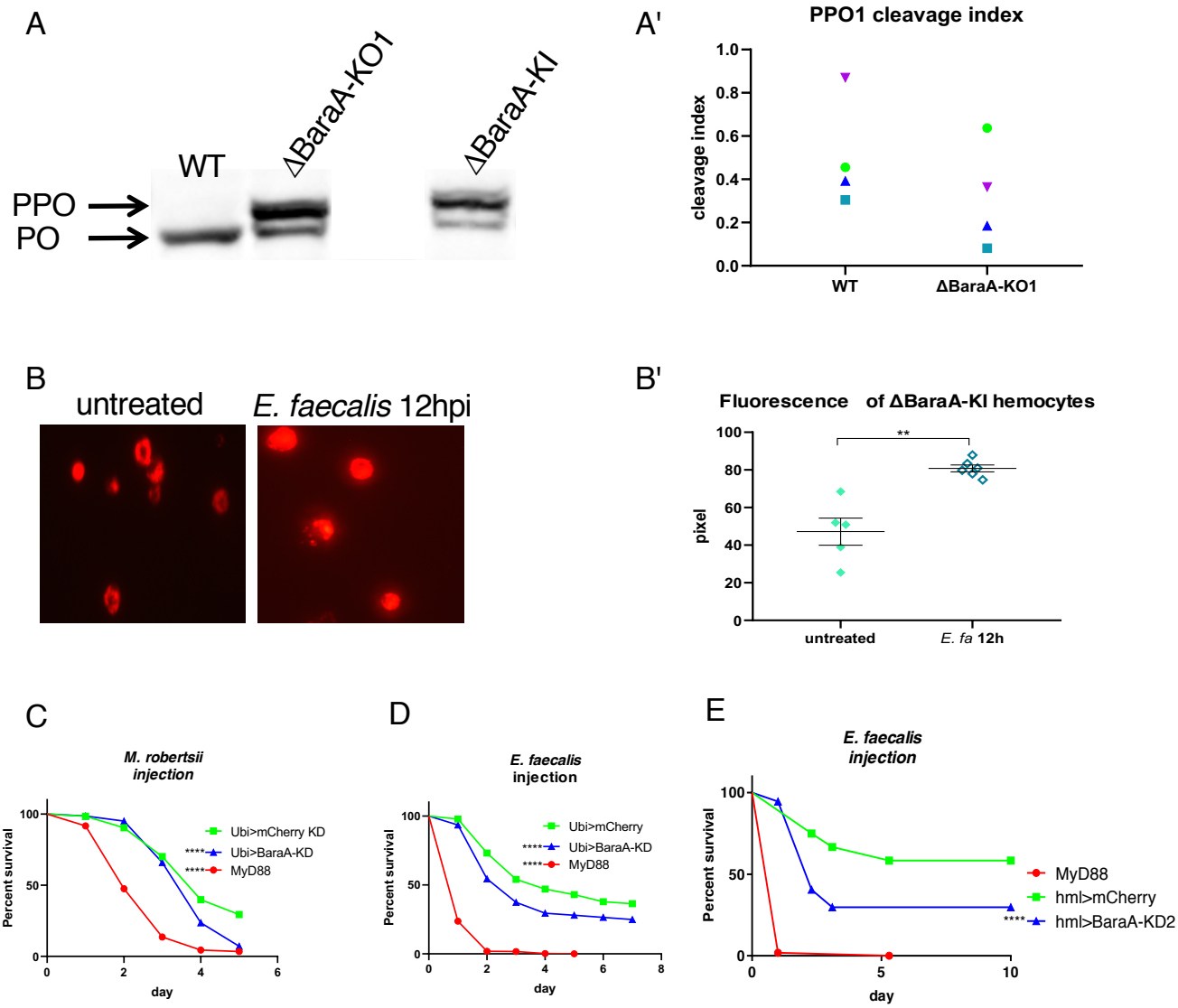

Fig. S4 *BaraA* may contribute to resistance against *E. faecalis* infection.
(A) The cleavage of prophenoloxidase was analyzed 4h after *M. luteus* septic injury by Western blotting using an anti-PPO1 antibody. **(A')** Quantification of the cleavage ratio of PPO1 in control and *BaraA KO1* flies in four independent experiments. Different colors correspond to different experiments; the purple triangles correspond to the experiment shown in Panel A in which PPO1 cleavage was nearly complete. The cleavage of PPO1 was stronger in wild-type than in *BaraA* KO1 in three out of four experiments. **(B)** Hemolymph was collected from adult *BaraA-mCherry* knock-in flies injected with *E. faecalis* for 12 hours or from untreated flies and the hemocytes were observed by fluorescence microscopy at 40X magnification. **(B')** Quantification of the fluorescence of hemocytes of *ΔBaraA*-KI displayed in (B). **(C)** Survival of *Ubi Gal4>UAS-BaraA* RNAi KD after an immune challenge with *M. robertsii*. Four out of seven experiments showed significant difference between BaraA-KD and control. Pooled data from seven independent experiments. ****P < 0.0001. **(D, E)** Survival of *Ubi Gal4>UAS-BaraA* RNAi KD (D) and *hmlGal4>UAS-BaraA* RNAi KD2 (E) after an immune challenge with *E. faecalis*. Statistical significance between wild type and KD flies (five out of five experiments in (D), three out of four experiments in (E)). LogRank test on pooled data, * p<0.05, ** p < 0.01, ****P < 0.0001.

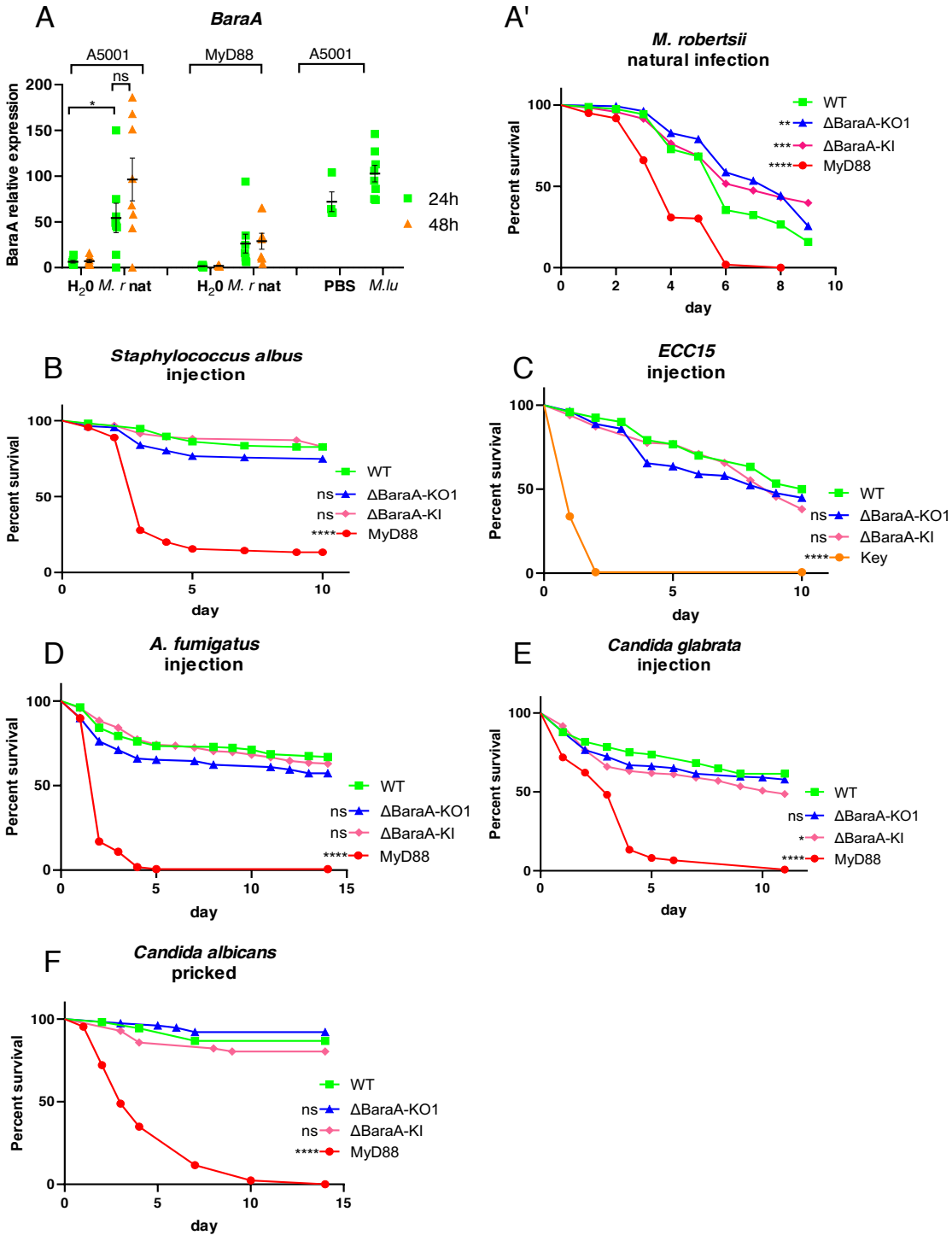

Figure S5: *BaraA* mutants survive as well as wild-type (WT) flies to different types of infection.

(A-A') Expression of *BaraA* steady-state transcripts as measured by RTqPCR (A) and survival after a natural *M. robertsii* infection challenge (A'). **(B-F)** Survival experiments after the indicated infectious challenge in the septic injury model are presented. The appropriate controls for the different microbes have been used: Gram-positive bacteria, fungi: *MyD88*, mutant of the Toll pathway; Gram-negative bacteria: *key*, mutant of the Immune deficiency pathway. None of the *BaraA* mutants displayed a reproducible susceptibility or resistance to infection (KO, KI). We used the log-rank test to determine the significance between wild-type and mutant survival curves. (A', F) Pooled data from at least two independent experiments. * $p<0.05$, **** $p<0.0001$. Data are expressed as means ± SEM.

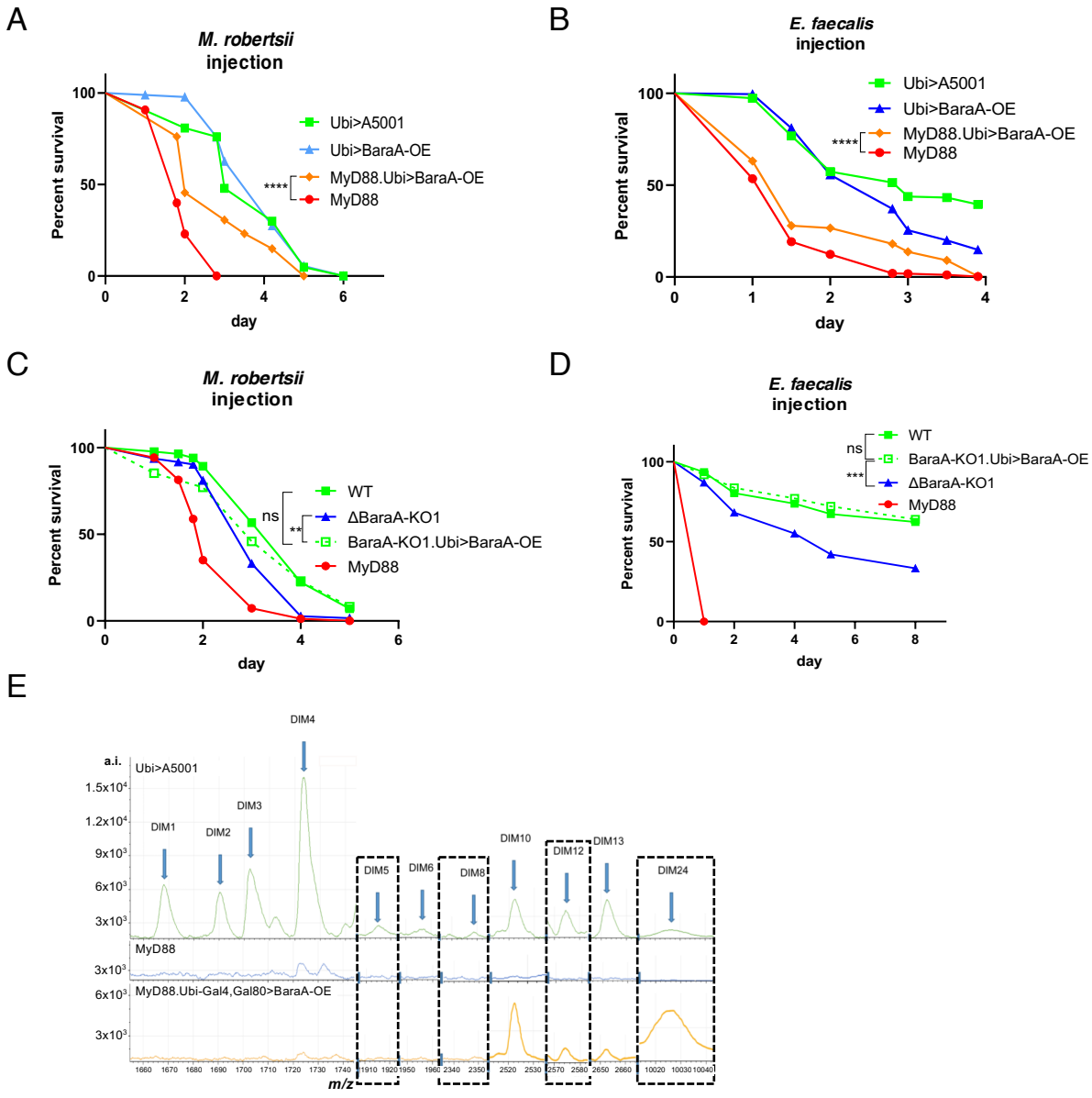

Figure S6: *BaraA* overexpression in *MyD88* but not in WT background confers an enhanced protection against *E. faecalis* and *M. robertsii* infection *in vivo*.

(A-B) BaraA overexpression in *MyD88* but not in WT background enhanced the protection against *M. robertsii* (A) and *E. faecalis* OG1RF (B) infection compared respectively to *MyD88* and WT flies. Pooled data from three independent experiments, **** $p<0.0001$. **(C-D)** Rescue of the nonisogenic *BaraA* KO1 mutant with a *BaraA* transgene expressed under the control of a p*Ubi*-Gal4^{ts} driver (cross performed at 18°C and induced at the adult stage at 29°C) after *M. robertsii* (C) or *E. faecalis* OG1RF injection (D). Three independent experiments with each pathogen have been performed and pooled. *$p < 0.05$. **(E)** Mass-spectra of hemolymph from single flies was collected 24h after a *M. luteus* challenge for wild-type control flies, *MyD88* or flies overexpressing *BaraA* in a *MyD88* mutant background. In *MyD88* flies, only DIM4 (Daisho) was slightly expressed in contrast to the wild-type control in which DIM12, DIM10, DIM13 and DIM24 were detected, as well as other DIMs. Only the relevant parts of the spectra are shown.

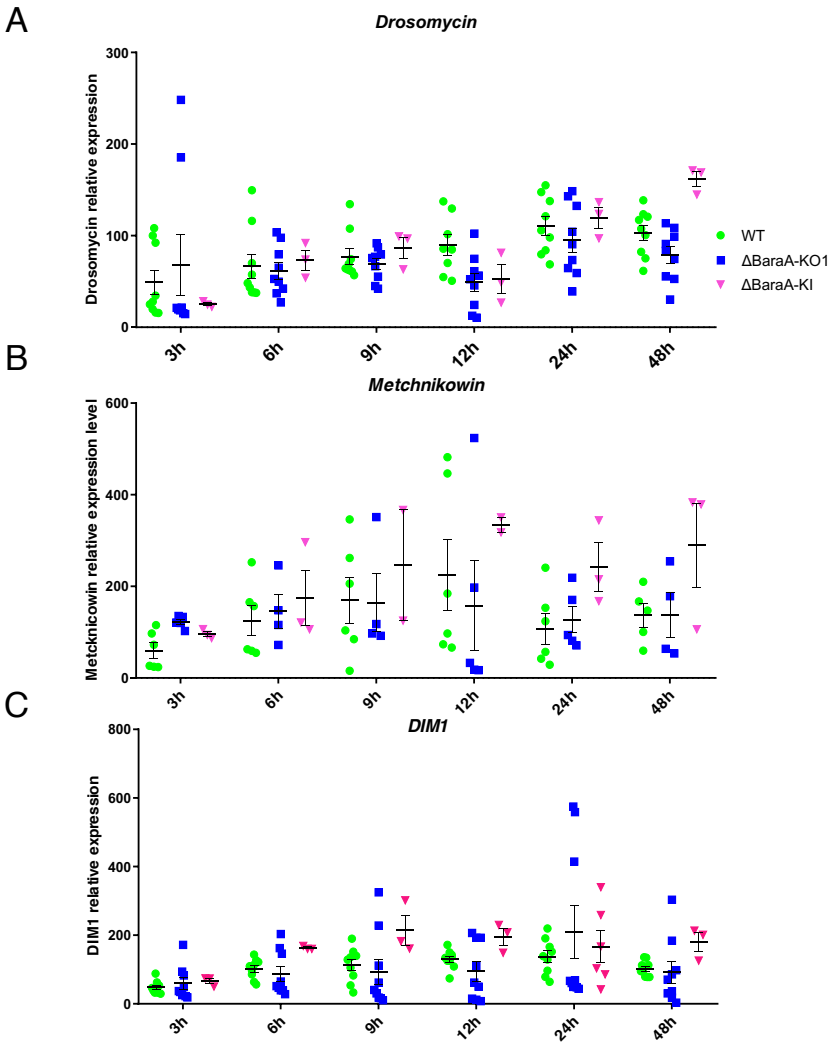

Figure S7. Toll-mediated activation of some of its target effector genes is not altered in *BaraA* KO or KI flies.

Steady-state transcript levels of *D. melanogaster* Toll pathway-regulated genes were measured by quantitative RT-PCR at different time points after a *E. faecalis* challenge: *Drosomycin* (A), *Metchnikowin* (B), and *DIM1=BomS1* (C). These experiments are representative of three independent experiments. Gene expression was normalized against *rpl32* gene expression and the results are normalized to the expression at 48h measured with WT. No significant difference between WT and isogenic *BaraA* mutants was detected. The Kruskall-Wallis multiple comparisons test has been used. Data are expressed as means ± SEM.

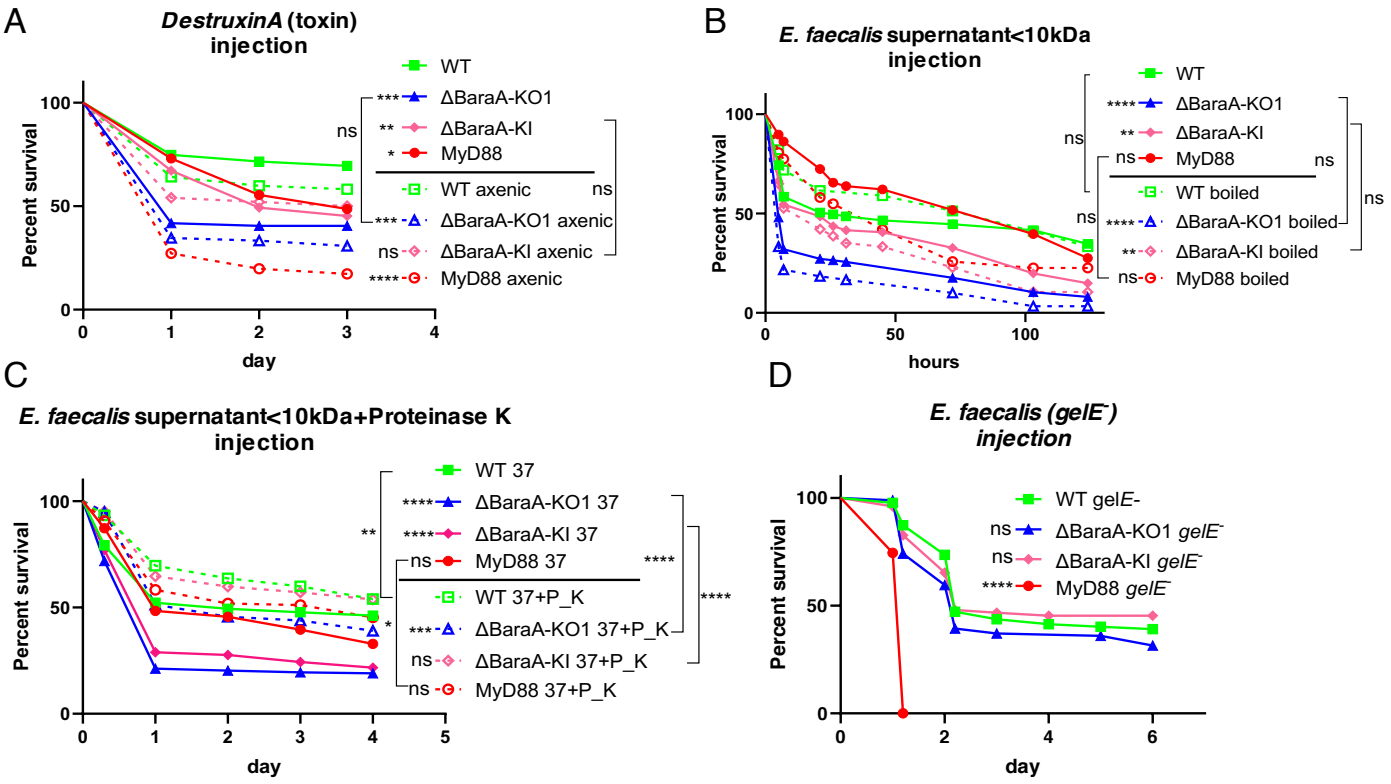

Figure S8. Further characterization of toxins secreted by *M. robertsii* or *E. faecalis*

(A) 4.6nL of 8mM DestruxinA were injected into axenic (dashed lines) or conventionally-raised flies. Axenic *BaraA* mutants showed no significant (ns) difference from control conventionally-raised *BaraA* mutant flies. Pooled data from two independent experiments, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001: comparison of mutant flies to WT flies for each condition. **(B)** Supernatant from *E. faecalis* OG1RF was boiled at 95°C for 5 min. Flies were injected with 23nl of the boiled (dashed lines) or untreated supernatant. For both conditions, injected mutant flies were significantly more susceptible than control WT flies. For each condition (above or below the line in the caption), mutant flies are compared to wild-type flies submitted to the same challenge for statistical analysis. Pooled data from two independent experiments, ** p< 0.01, **** p<0.0001. **(C)** Supernatant was incubated with 100ug/ml Proteinase K or with PBS (the same volume as Proteinase K) at 37°C for 18 hours. 23nl of supernatant was injected. *BaraA*-KO1 with Proteinase K treated supernatant (dashed lines) died slower than the flies injected with untreated supernatant. The same result was observed for *BaraA*-KI. Pooled data from seven independent experiments, * p<0.05, ** p< 0.01, *** p<0.001, **** p< 0.0001. **(D)** 4.6nL of 0.5 OD of the *GelE- E. faecalis* mutant strain was injected. No statistically significant difference was observed. Pooled data from five independent experiments.

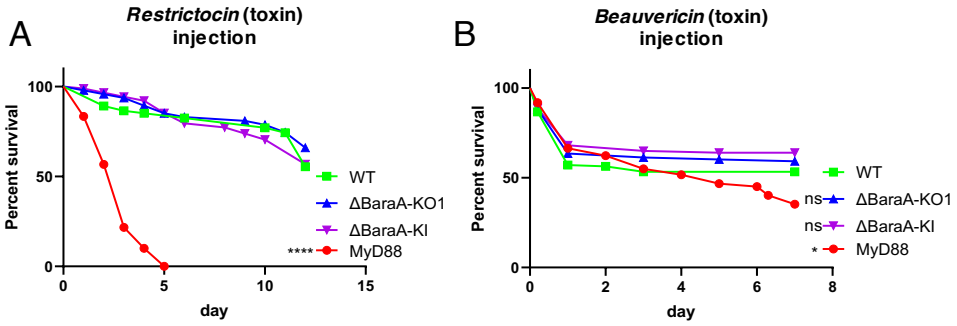

Fig. S9 BaraA is specifically counteracting toxins.

(A) Survival of *BaraA* mutants following Restrictocin injection. No statistically significant difference between wild type flies and *BaraA* mutant flies was observed. Pooled data from two independent experiments. **(B)** Upon Beauvericin injection (toxin from *Beauveria bassiana*), *MyD88* and not *BaraA* mutants displayed sensitivity to this challenge. Pooled data from two independent experiments, * $p<0.05$.

Table S1: MS results of hemolymph collected from *BaraA* mutant flies 24h after *M. luteus* challenge

	Average molecular masse (*m/z*)		Wild type		ΔBaraA-KO1		ΔBaraA-KI	
	Theroretical	Measured	Peak area	Peak intensity	Peak area	Peak intensity	Peak area	Peak intensity
DIM1(BomS1)	1667	1668	67258	16767	62061	12083	103407	21980
DIM2(BomS2)	1690	1690	40706	11767	61520	11580	82204	16824
DIM3(BomS3)	1704	1703	80236	20008	72387	15006	121265	25515
DIM4(Dso1)	1723	1724	134713	26255	260151	44055	289787	52637
DIM5	1914	1915	47815	15539	1863 (0)	2276 (0)	3125 (0)	5929 (0)
DIM6	1955	1957	121705	30592	-	-	-	-
DIM8	2348	2349	46950	18659	2182 (0)	1672 (0)	-	-
DIM10	2521	2523	257018	51458	2738	1797	9619	6569
DIM12	2573	2575	165811	36743	-	-	-	0
DIM13	2651	2653	355175	68504	10071 (0)	2287 (0)	-	6020
DIM24	10031	10034	141464	4599	-	-	-	-
Drosomycin (Drs)	4890	4892	2065642	168320	155898	13213	639405	50870

Quantitation of Fig. S3
(0) correspond to signals almost undistinguishable from the background

Table S2: MS results of hemolymph collected on *BaraA*-overexpressing *MyD88* flies 24h after *M. luteus* challenge

	Average molecular masse (*m/z*)		Ubi>A5001		MyD88		BaraA-OE (MyD88)	
	Theroretical	Measured	Peak area	Peak intensity	Peak area	Peak intensity	Peak area	Peak intensity
DIM1(BomS1)	1667	1668	39814	5291	2262	283	-	105
DIM2(BomS2)	1690	1691	36729	4492	-	111	-	136
DIM3(BomS3)	1704	1703	70522	10659	1113	283	-	108
DIM4(Dso1)	1723	1724	120283	15820	6007	1337	2387	510
DIM5	1914	1915	6829	1223	-	-	-	-
DIM6	1955	1957	10952	1874	-	-	-	-
DIM8	2348	2349	13152	2049	-	-	531	175
DIM10	2521	2523	154384	20693	685	164	26381	4382
DIM12	2573	2575	104939	14020	-	-	4563	926
DIM13	2651	2653	169357	24429	-	-	4211	828
DIM24	10031	10033	29166	1054	-	-	134314	4227
Drosomycin (Drs)	4890	4892	184661	14427	9057	977	7168	844

Quantitation of Fig. S6E

Table S3: Primers used for cloning

BaraA Fw	AAAAAGCAGGCTTCAACATGAAATCGTTTGGATTGATTGC
BaraA Rv	AGAAAGCTGGGTCTTAAACTTTTTGGAGGCATATGA
BaraA Rv-HA	AGAAAGCTGGGTCAACTTTTTGGAGGCATATGA
2nd-F	GGGGACAAGTTTGTACAAAAAAGCAGGCT
2nd-R	GGGGACCACTTTGTACAAGAAAGCTGGGT
BaraA gRNA-F(KI)	GCGGCCCGGGTTCGATTCCCGGCCGATGCAATCTGTGGCGT TATCTGCGTGTTTTAGAGCTAGAAATAGCAAG
BaraA gRNA-R(KI)	ATTTTAACTTGCTATTTCTAGCTCTAAAACCTGCCTTTTACA ACACTGCATGCACCAGCCGGGAATCGAACCC
BaraA gRNA	ACCCACTCCCGGCACGCTGT
CG18278 gRNA	ACCACACTGGCGGCGAGCGC

Table S4: Primers used for RTqPCR

RpL32 Fw	GCTAAGCTGTCGCACAAATG
RpL32 Rv	GTTCGATCCGTAACCGATGT
Drosomycin Fw	TACTTGTTCGCCCTCTTCG
Drosomycin Rv	GAGCGTCCCTCCTCCTTGC
IM1 Fw	CAATGCTGTTCCACTGTCGC
IM1 Rv	CGTGGACATTGCACACCCTG
Metchnikowin Fw	CGTCACCAGGGACCCATTT
Metchnikowin Rv	CCGGTCTTGGTTGGTTAGGA
BaraA Fw	GGTGAGCATGTGTACACCGA
BaraA Rv	GGCGGAAAAATTGGGACCAC
CG18278 Fw	GCCCATGAACCCTTCACTCC
CG18278 Rv	CACCAACCAGTGCTTATCCTGC