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Supplemental Information

The Circulating Protease Persephone Is an Immune Sensor for Microbial Proteolytic Activities Upstream of the *Drosophila* Toll Pathway

Najwa Issa, Nina Guillaumot, Emilie Lauret, Nicolas Matt, Christine Schaeffer-Reiss, Alain Van Dorsselaer, Jean-Marc Reichhart, and Florian Veillard

Figures S1 to S5

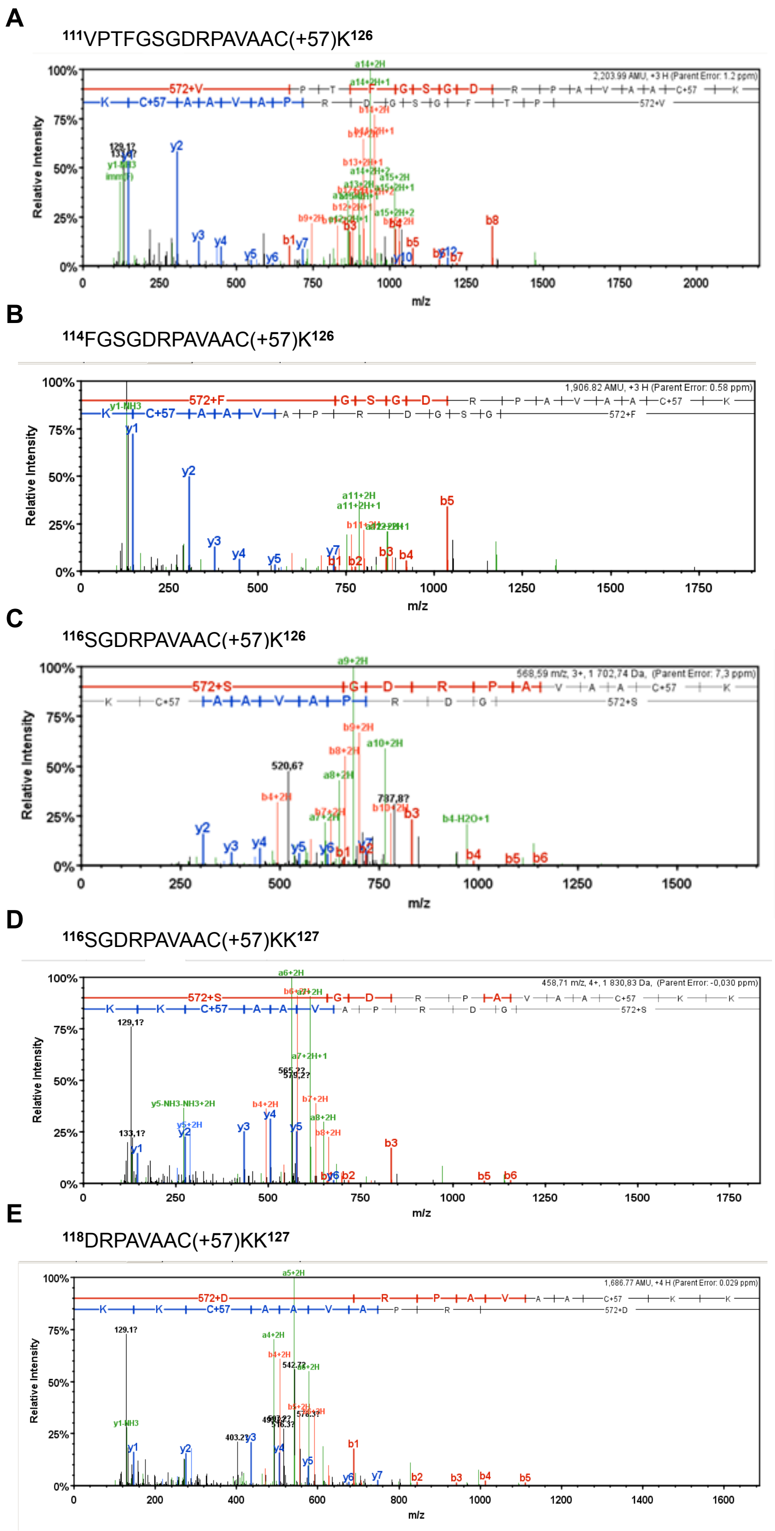

Figure 1 : MS/MS spectra of the N-terminal peptides identified using in-gel protein strategy after proteolytic maturation (Related to Figure 3)
(A) *E. faecalis* and *S. marcescens*, (B) *P. aeruginosa*, (C) *E. faecalis*, (D) *S. aureus* and *B. bassiana* and (E) *S. aureus*.

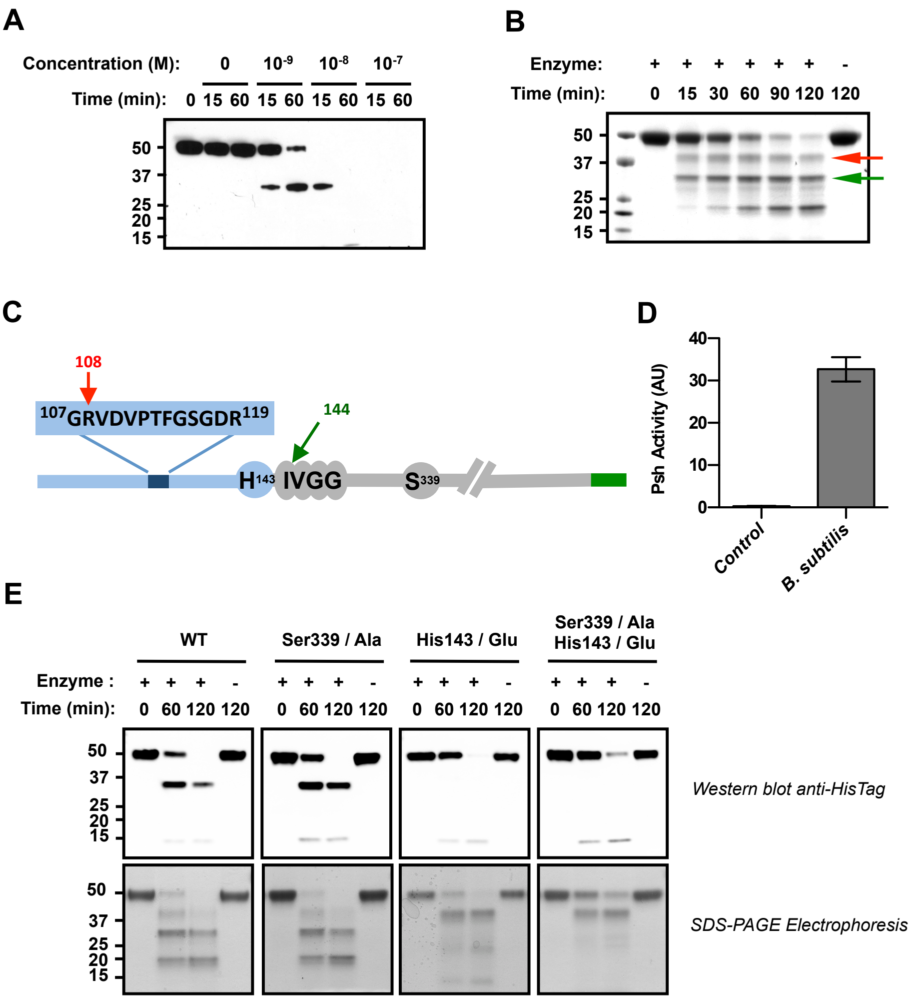

Figure S2: Specific activation of Psh by *B. subtilis* subtilisin (Related to Figure 3)

(A) rpro-Psh (0.2 µg/µl) was incubated at 29 °C with various concentrations of *B. subtilis* purified subtilisin. After 15 or 60 min, 1 µg aliquots were removed and boiled for 5 min to stop the reaction. After SDS-PAGE electrophoresis, residual rpro-Psh was visualized using anti-HisTag antibody. (B) rpro-Psh (0.2 µg/µl) was incubated at 29 °C with *B. subtilis* (1 nM) purified protease. At various time points, 5 µg aliquots were removed and boiled for 5 min to stop the reaction. Samples were then electrophoresed and stained with Coomassie blue. Representative results of at least 2 independent experiments. (C) The N-terminal extremities of the main hydrolysis products (indicated by arrows) were determined by nanoLC-MS/MS analysis after in-gel protein N-terminal labeling using TMPP-Ac-Osu. (D) Cell-free supernatant of S2 cells expressing rpro-Psh (200 µl) was incubated in TrisHCl buffer 0.1 M, pH 8 with *B. subtilis* (1 nM) protease. After 1 hour, proteolytic activity of the generated rPro-Psh hydrolysis products was determined on the fluorogenic substrate Z-Arg-AMC for 30 min at 29 °C in 0.1 M TrisHCl buffer pH 8 supplemented with 5 mM $CaCl_2$. (E) rpro-Psh mutants His143/Glu, Ser339/Ala and His143/Glu; Ser339/Ala (0.2 µg/µl) were incubated with *B. subtilis* protease under the same conditions. After 1 or 2 hours, residual proteins were observed by Western blot with anti-6HisTag antibody and hydrolysis products visualized by Coomassie blue staining.

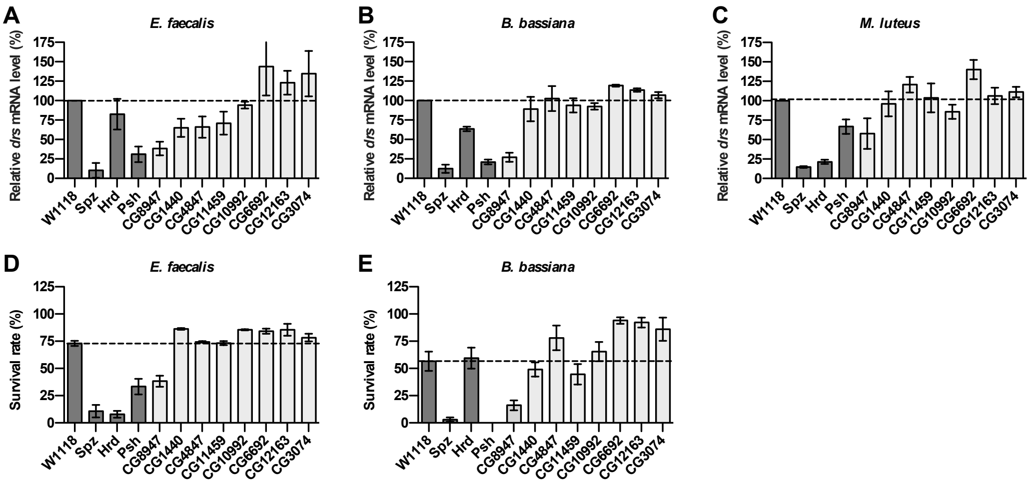

Figure S3: Implication of cysteine cathepsins in the Toll pathway (Related to Figure 5)

(A-C) Flies deficient for the indicated gene (See Sup Table 1) were challenged by septic injury with *E. faecalis* (OD_{600}=1) (A), *M. luteus* (OD_{600}>200) (C) or by natural infection with *B. bassiana* (B). After 24 hours at 29 °C, flies were collected and *drs* gene expression was monitored by RT-qPCR in total RNA extracts. *Ribosomal protein 49* (*Rp49*) mRNA was used as reference gene. Results were normalized to the value obtained for w^{1118} control flies. Data represent means ± standard errors of 3 independent experiments, each containing three groups of 10 flies (5 males and 5 females). (D-E) Survival rate of adult flies challenged with *E. faecalis* by septic injury (OD_{600}=1) or with *B. bassiana* by natural infection 72 hours post-infection. Results are normalized with control flies (w^{1118} flies for null mutants and *C564-gal4* flies for RNAi expressing flies). Data represent means ± standard errors of 3 independent experiments, each containing three groups of 20 flies (10 males and 10 females).

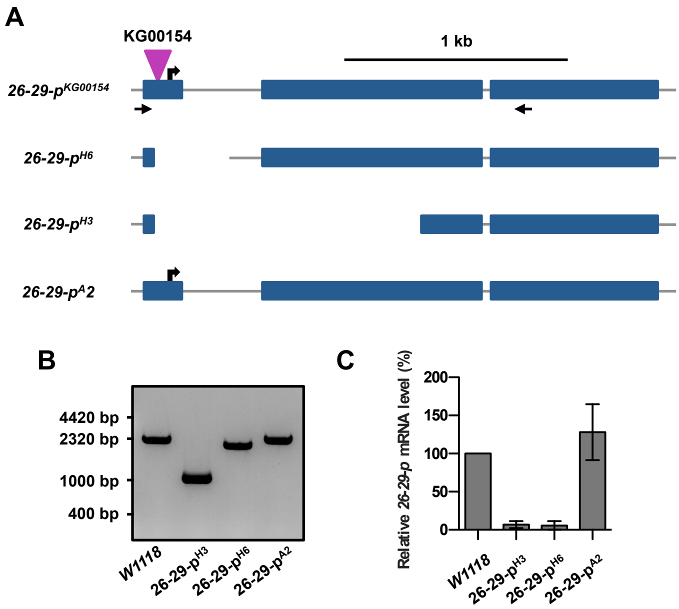

Figure S4: Generation of cathepsin 26-29-p mutants (Related to Figure 6)
(A) Schematic representation of the genomic region of *26-29-p* and the mutant alleles obtained by P-element excision. Two null mutants and one revertant fly lines were generated by excision of the *KG00154* P-element (purple arrow) following crosses with *P(Δ2-3)* transposase flies (Bloomington #2534). (B) PCR products obtained using the primers forward: 5'-GTCCGACTATCGGTTCGGTTT-3' and reverse: 5'-GATTGCCGCCATTCTTCAGG-3' and indicated by black arrows in (A). (C) Flies were collected and *26-29-p* gene expression was monitored by RT-qPCR in total RNA extracts. *Ribosomal protein 49* (*Rp49*) mRNA was used for normalization. Data represent means ± standard errors of 3 independent experiments, each containing three groups of 10 flies (5 males and 5 females).

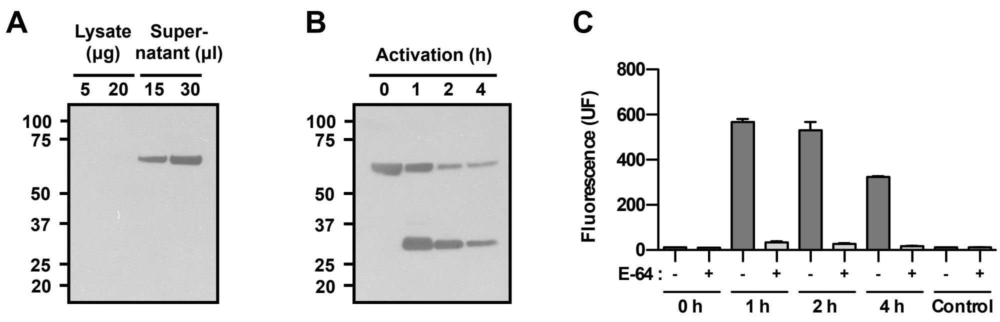

Figure S5: Expression and activation of rpro-cathepsin 26-29-p (Related to Figure 7)

S2 cells were stably transfected with the expression plasmid of rpro-cathepsin 26-29-p as described previously. (A) Expression of rpro-cathepsin 26-29-p was assessed in S2 cells lysate and cells culture supernatant by Western blot using the monoclonal Anti-6His C-term antibody. (B) To activate rpro-cathepsin 26-29-p, cells culture media was concentrated 20 times and then incubated with pepsine (0.002 mg/ml) in 0.2 M Glycine buffer, pH 4. After incubation at 37 °C, rpro-cathepsin 26-29-p processing was followed by Western blot with the monoclonal Anti-6His C-term antibody. (C) Activity of the generated hydrolysis products was assessed with or without E-64 (0.1 mM) at 37 °C in 0.1 M sodium acetate buffer, pH 5.5 on the fluorogenic substrate Z-Phe-Arg-AMC (λ_{ex} = 350 nm; λ_{em} = 460 nm). Pepsine alone was used as control.

Supplementary Table S1 TO S5

Table S1: List of Plasmids (Related to STAR Methods)

Plasmid name	Construction	Destination plasmid	Details
pJM1345	rpro-Psh	pMT-V5	PCR on cDNA clone GH12385 (DGRC) with primers IMU839/840 cloned in KpnI-XhoI
pJM1681	rpro-Psh^{M1}	pMT-V5	PCR on pJM1345 with primers IMU1144/1342, IMU1341/1145, IMU1144/1145 cloned in BglII-XhoI
pJM1682	rpro-Psh^{M2}	pMT-V5	PCR on pJM1345 with primers IMU 1343/1344 cloned in Bgl2-XhoI
pJM1692	pro-Psh	pUAST-ATTB	KpnI-PmeI fragment from pJM1345
pJM1693	pro-Psh^{M1}	pUAST-ATTB	KpnI-PmeI from fragment pJM1681
pJM1694	pro-Psh^{M2}	pUAST-ATTB	KpnI-PmeI fragment from pJM1682
pJM1674	rpro-Psh Ser339 / Ala	pMT-V5	PCR on pJM1345 with primers IMU1144/1228, IMU1145/1229, IMU1144/1145 cloned in KpnI-XhoI
pJM1675	rpro-Psh His144 / Glu	pMT-V5	PCR on pJM1345 with primers IMU1144/1338, IMU1145/1337, IMU1144/1145 cloned in KpnI-XhoI
pJM1676	rpro-Psh His143 / Glu ; Ser339 / Ala	pMT-V5	PCR on pJM674 with primers IMU1144/1338, IMU1145/1337, IMU1144/1145 cloned in KpnI-XhoI
pJM1696	rpro-cathepsin 26-29-p	pMT-V5	PCR on cDNA clone pRE 18380 (DGRC) with primers T7/IMU1347 cloned in EcoR1-Apa1

Table S2: List of Primers for PCR (Related to STAR Methods)

Primer name	Sequence (5'-3')
IMU 839	GGGGGGTACCAAGATGCCATTGAAGTGGTCCCTGC
IMU 840	GGGGCTCGAGCACCCGATTGTCCGGCCAGA
IMU 1144	TGTGGTCAGCAGCAAAATCAAGTG
IMU 1145	CTGCATTCTAGTTGTGGTTTGTCC
IMU 1228	GCTCATGAATGAGCGGCCCACCGGCGTCGCCCTTGCATGCGTCGGCG
IMU 1229	CGCCGACGCATGCAAGGGCGACGCCGGTGGGCCGCTCATTCATGAGC
IMU 1337	GAGCGGCAATCAATTGGTCATAGACATCGTGGGCGGTTATCC
IMU 1338	GGATAACCGCCCACGATGTCTATGACCAATTGATTGCCGCTC
IMU 1341	GCTGCTGCTGCTGCTCCCACGTTCGGAAGCGGT
IMU 1342	AGCAGCAGCAGCAGCACTGGTCATTGGAGCTTTTGTGC
IMU 1343	GCTGCTGCTGCTGCTGCTGCTGCTGCTAGCGGTGATCGCCCAGC
IMU 1344	AGCAGCAGCAGCAGCAGCAGCAGCAGCACTGGTCATTGGAGCTTTTGTGC
IMU 1345	GCACAAAAGCTCCAATGACCAGTAGCGGTGATCGCCCAGC
IMU 1346	GCTGGGCGATCACCGCTACTGGTCATTGGAGCTTTTGTGC
IMU 1347	GCTTACCTTCGAAGGGCCCCATCTCCACATAAGTGGGCATGG
T7	TGTAAAACGACGGCCAGTGA

Table S3: Screening of cysteine cathepsins (Related to STAR Methods)

Name	**CG number**	**Bloomington stock number**	**Genotype**
26-29-p	CG8947	13051	P(SUPor-P)26-29-p^{KG00154}
Cathepsin L1	CG6692	32932	P(TRIP.HMS00725)attP2
CG4847	CG4847	42655	P(TRIP.HMS02491)attP2
CG12163	CG12163	33955	P(TRIP.HMS00910)attP2
CG11459	CG11459	50488	Mi(MIC)CG11459^{MI08810}
Cathepsin B1	CG10992	15434	P(EPgy2)CtsB1^{EY03339}
Swing	CG3074	36591	P(TRIP.GL00551)attP2
Bleomycin Hydrolase	CG1440	13977	P(SUPor-P)CG1440^{KG04580}

Table S4: Ms/Ms fragmentation table of the N-terminal TMPP labeled peptide IVGGYPVDPGVYPHMAAIGYITFGTDFR (Mascot interpretation, Related to Figure 3).

amino acid	b ion	a (M)	a* (M)	a*++ (M)	a++ (M)	a0 (M)	a0++ (M)	b (M)	b++ (M)	b0 (M)	b0++ (M)	y (M)	y* (M)	y*++ (M)	y++ (M)	y0 (M)	y0++ (M)	y ion
I	1	**658.2776**	641,251	321,1291	329,6424	640,267	320,6371	**686.2725**	343,6399									28
V	2	**757.346**	740,3194	370,6634	379,1766	739,3354	370,1713	**785.3409**	393,1741			2916,403	2899,376	1450,192	1458,705	2898,392	1449,7	27
G	3	814,3674	797,3409	399,1741	407,6874	796,3569	398,6821	842,3624	421,6848			2817,334	2800,308	1400,657	1409,171	2799,324	1400,165	26
G	4	**871.3889**	854,3624	427,6848	436,1981	853,3783	427,1928	**899.3838**	450,1955			2760,313	2743,286	1372,147	1380,66	2742,302	1371,655	25
Y	5	**1034.4522**	1017,426	509,2165	517,7298	1016,442	508,7245	**1062.4471**	531,7272			2703,291	2686,265	1343,636	1352,149	2685,281	1343,144	24
P	6	1131,505	1114,478	557,7429	566,2561	1113,494	557,2509	1159,5	580,2536			2540,228	2523,201	1262,104	1270,618	2522,217	1261,612	23
V	7	**1230.5734**	1213,547	607,2771	615,7903	1212,563	606,7851	**1258.5683**	629,7878			2443,175	2426,149	1213,578	1222,091	2425,165	1213,086	22
D	8	1345,6	1328,574	664,7905	673,3038	1327,59	664,2985	**1373.5953**	**687.3013**	1355,585	678,296	2344,107	2327,08	1164,044	1172,557	2326,096	1163,552	21
P	9	1442,653	1425,627	713,3169	721,8302	1424,643	712,8249	1470,648	735,8277	1452,638	726,8224	**2229.0797**	2212,053	1106,53	**1115.0435**	2211,069	1106,038	20
G	10	1499,675	1482,648	741,8277	750,3409	1481,664	741,3356	**1527.6695**	764,3384	1509,659	755,3331	**2132.027**	2115	1058,004	1066,517	2114,016	1057,512	19
V	11	1598,743	1581,716	791,3619	799,8751	1580,732	790,8699	**1626.7379**	813,8726	1608,727	804,8673	2075,006	2057,979	1029,493	1038,006	2056,995	1029,001	18
Y	12	1761,806	1744,78	872,8935	881,4068	1743,796	872,4015	**1789.8012**	895,4043	1771,791	886,399	**1975.9371**	1958,911	979,9589	**988.4722**	1957,927	979,4669	17
P	13	1858,859	1841,833	921,4199	929,9332	1840,849	920,9279	1886,854	943,9306	1868,843	934,9254	**1812.8738**	1795,847	898,4272	906,9405	1794,863	897,9352	16
H	14	1995,918	1978,891	989,9494	998,4626	1977,907	989,4574	2023,913	1012,46	2005,902	1003,455	1715,821	1698,795	849,9009	858,4141	1697,81	849,4089	15
M	15	2142,953	2125,927	**1063.4671**	1071,98	2124,943	1062,975	2170,948	1085,978	2152,938	1076,973	**1578.7621**	1561,736	781,3714	789,8847	1560,752	780,8794	14
A	16	2213,991	2196,964	1098,986	1107,499	**2195.9799**	1098,494	**2241.9854**	1121,496	2223,975	1112,491	**1431.7267**	1414,7	707,8537	716,367	1413,716	707,3617	13
A	17	2285,028	2268,001	1134,504	1143,018	2267,017	1134,012	2313,023	1157,015	2295,012	1148,01	**1360.6896**	1343,663	672,3352	680,8484	1342,679	671,8431	12
I	18	2398,112	2381,085	1191,046	1199,56	2380,101	1190,554	2426,107	1213,557	2408,096	1204,552	**1289.6525**	1272,626	636,8166	645,3299	1271,642	636,3246	11
G	19	2455,133	2438,107	1219,557	1228,07	2437,123	1219,065	2483,128	1242,068	2465,118	1233,062	**1176.5684**	1159,542	580,2746	588,7878	1158,558	579,7826	10
Y	20	2618,197	2601,17	1301,089	1309,602	2600,186	1300,597	2646,191	1323,599	2628,181	1314,594	**1119.5469**	1102,52	551,7638	560,2771	1101,536	551,2718	9
I	21	2731,281	2714,254	1357,631	1366,144	2713,27	1357,139	2759,276	1380,141	2741,265	1371,136	**956.4836**	939,4571	470,2322	478,7454	938,473	469,7402	8
T	22	2832,328	2815,302	1408,155	1416,668	2814,318	1407,663	2860,323	1430,665	2842,313	1421,66	**843.3995**	826,373	413,6901	422,2034	**825.389**	413,1981	7
F	23	2979,397	2962,37	1481,689	1490,202	2961,386	1481,197	3007,392	1504,199	2989,381	1495,194	**742.3519**	725,3253	363,1663	371,6796	724,3413	362,6743	6
G	24	3036,418	3019,392	1510,199	1518,713	3018,408	1509,707	3064,413	1532,71	3046,403	1523,705	**595.2835**	578,2569	289,6321	298,1454	577,2729	289,1401	5
T	25	3137,466	3120,439	1560,723	1569,237	3119,455	1560,231	3165,461	1583,234	3147,45	1574,229	**538.262**	521,2354	261,1214	269,6346	520,2514	260,6293	4
D	26	3252,493	3235,466	1618,237	**1626.75**	3234,482	1617,745	3280,488	1640,748	3262,477	1631,742	**437.2143**	420,1878	210,5975	219,1108	419,2037	210,1055	3
F	27	3399,561	3382,535	1691,771	1700,284	3381,551	1691,279	3427,556	1714,282	3409,546	1705,276	**322.1874**	**305.1608**	153,084	161,5973			2
R	28											**175.119**	158,0924	79,5498	88,0631			1

Table S5: Log-rank analyses of flies survival assays (OASIS online application) (Related to Figure 6)

	B. bassiana			***E. faecalis***		
Condition	**Chi^2**	**P-value**	**Bonferroni P-value**	**Chi^2**	**P-value**	**Bonferroni P-value**
w^{1118} *v.s.* ***Psh***	78.36	0.0e+00	0.0e+00	16.63	4.5e-05	0.0002
w^{1118} *v.s.* ***Spz***	79.56	0.0e+00	0.0e+00	62.94	0.0e+00	0.0e+00
w^{1118} *v.s.* ***26-29-p^{H3}***	84.16	0.0e+00	0.0e+00	14.72	0.0001	0.0006
w^{1118} *v.s.* ***26-29-p^{H6}***	67.11	0.0e+00	0.0e+00	9.70	0.0018	0.0092
w^{1118} *v.s.* ***26-29-p^{A2}***	1.83	0.1758	0.8792	0.00	0.9505	1.0000
Psh *v.s.* ***w^{1118}***	78.36	0.0e+00	0.0e+00	16.63	4.5e-05	0.0002
Psh *v.s.* ***Spz***	5.32	0.0211	0.1056	32.07	1.5e-08	7.5e-08
Psh *v.s.* ***26-29-p^{H3}***	2.52	0.1122	0.5610	0.01	0.9195	1.0000
Psh *v.s.* ***26-29-p^{H6}***	2.34	0.1261	0.6304	0.65	0.4214	1.0000
Psh *v.s.* ***26-29-p^{A2}***	49.70	0.0e+00	0.0e+00	17.63	2.7e-05	0.0001
Spz *v.s.* ***w^{1118}***	79.56	0.0e+00	0.0e+00	62.94	0.0e+00	0.0e+00
Spz *v.s.* ***Psh***	5.32	0.0211	0.1056	32.07	1.5e-08	7.5e-08
Spz *v.s.* ***26-29-p^{H3}***	0.18	0.6751	1.0000	24.51	7.4e-07	3.7e-06
Spz *v.s.* ***26-29-p^{H6}***	8.90	0.0028	0.0142	29.92	4.5e-08	2.3e-07
Spz *v.s.* ***26-29-p^{A2}***	54.91	0.0e+00	0.0e+00	66.53	0.0e+00	0.0e+00
26-29-p^{H3} *v.s.* ***w^{1118}***	84.16	0.0e+00	0.0e+00	14.72	0.0001	0.0006
26-29-p^{H3} *v.s.* ***Psh***	2.52	0.1122	0.5610	0.01	0.9195	1.0000
26-29-p^{H3} *v.s.* ***Spz***	0.18	0.6751	1.0000	24.51	7.4e-07	3.7e-06
26-29-p^{H3} *v.s.* ***26-29-p^{H6}***	6.96	0.0083	0.0416	0.61	0.4351	1.0000
26-29-p^{H3} *v.s.* ***26-29-p^{A2}***	55.06	0.0e+00	0.0e+00	15.56	0.0001	0.0004
26-29-p^{H6} *v.s.* ***w^{1118}***	67.11	0.0e+00	0.0e+00	9.70	0.0018	0.0092
26-29-p^{H6} *v.s.* ***Psh***	2.34	0.1261	0.6304	0.65	0.4214	1.0000
26-29-p^{H6} *v.s.* ***Spz***	8.90	0.0028	0.0142	29.92	4.5e-08	2.3e-07
26-29-p^{H6} *v.s.* ***26-29-p^{H3}***	6.96	0.0083	0.0416	0.61	0.4351	1.0000
26-29-pH6 *v.s.* ***26-29-p^{A2}***	39.74	0.0e+00	0.0e+00	10.51	0.0012	0.0059
26-29-p^{A2} *v.s.* ***w^{1118}***	1.83	0.1758	0.8792	0.00	0.9505	1.0000
26-29-p^{A2} *v.s.* ***Psh***	49.70	0.0e+00	0.0e+00	17.63	2.7e-05	0.0001
26-29-p^{A2} *v.s.* ***Spz***	54.91	0.0e+00	0.0e+00	66.53	0.0e+00	0.0e+00
26-29-p^{A2} *v.s.* ***26-29-p^{H3}***	55.06	0.0e+00	0.0e+00	15.56	0.0001	0.0004
26-29-p^{A2} *v.s.* ***26-29-p^{H6}***	39.74	0.0e+00	0.0e+00	10.51	0.0012	0.0059