Lactobacillus plantarum **is a pathobiont for adult** ***Drosophila*****.**

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SUMMARY

Pathobionts are a unique class of symbionts that cause fulminant, and potentially life-threatening disease in animal hosts under specific circumstances. Despite the relevance of pathobiont containment for prolonged health, we know very little about the basis for pathobiont-dependent disease in an animal host. We found that mono-association of adult *Drosophila* with *Lactobacillus plantarum*, a widely reported fly commensal, and member of the probiotic *Lactobacillus* genus, curtails adult longevity and causes extensive intestinal pathology within the host. Intestinal disease includes widespread loss of progenitor cells, disrupted epithelial organization, and impaired barrier function. We show that this phenotype is reverted in flies mutant for the *immune deficiency* gene, a regulatory component of fly innate defenses against gut bacteria. Our study uncovers a previously unknown pathogenic aspect of *Lactobacillus plantarum* association with *Drosophila* and establishes a simple model for the mechanistic exploration of pathobiont biology.

INTRODUCTION

An unpredictable landscape of environmental inputs overlaps with the biochemical outputs of prokaryotic and eukaryotic genomes to shape the intestinal microbiome. Extrinsic factors such as oxygen and nutrient supply influence the biogeography of microbiome distribution, while bacteriostatic products such as antimicrobial peptides (AMP) and reactive oxygen species (ROS) prevent bacterial dissemination throughout the host. Combined, these interactions lead to colonization of the metazoan gut by a shifting community of microbes. The metabolic output from this community influences critical events in their host, with profound implications for processes as diverse as growth, immunity, and behavior (Hacquard et al., 2015, Hooper et al., 2012, Hsiao et al., 2013, Kamada et al., 2013a, Kamada et al., 2013b, Wong et al., 2014). This entente cordiale between a host and their prokaryotic symbionts is essential for the long-term health of an animal, as disrupted host-symbiont relationships often lead to the onset of debilitating and potentially deadly host diseases (Blumberg and Powrie, 2012, Carding et al., 2015, Kamada et al., 2013a).

Drosophila melanogaster is a particularly useful tool to study host-microbe relationships. As is often the case, flies lend themselves to sophisticated manipulations that allow investigators to control the expression of almost any gene in specific intestinal cell types (Lemaitre and Miguel-Aliaga, 2013). Of equal importance, there are extensive genetic, developmental, and biochemical similarities between key aspects of fly and mammalian gut biology (Buchon et al., 2013, Jiang and Edgar, 2012, Ma et al., 2015). Thus, discoveries in *Drosophila* have tremendous value for the illumination of foundational aspects of host-microbe interactions. From a physiological perspective, the posterior midgut of *Drosophila* is functionally similar to the small intestine of mammals. In both cases, the epithelium contains secretory enteroendocrine cells and absorptive enterocytes that arise from a transitory progenitor cell – the enteroblast of *Drosophila*, and the transitory amplifying cells of mammals (Lemaitre and Miguel-Aliaga, 2013).

Both types of progenitor are maintained by asymmetric divisions of basally located Intestinal Stem Cells (ISC) (Jiang and Edgar, 2012, Takashima and Hartenstein, 2012). In addition, flies and mammals rely on evolutionarily conserved innate immune defenses, ROS generation, and homeostatic intestinal proliferation to prevent bacterial dissemination throughout the host (Buchon et al., 2013).

Although the microbiome of *Drosophila* is several order of magnitudes less complex than that found in mammals (Broderick and Lemaitre, 2012), populations of *Lactobacillus* species are common to fly midguts and animal small intestines. Studies of the *Drosophila* commensal, *Lactobacillus plantarum* (*Lp*), uncovered a remarkable degree of interaction between the two species. *Lp* contributes to larval growth (Storelli et al., 2011), uptake of dietary protein (Erkosar et al., 2015), and management of malnutrition in the host (Schwarzer et al., 2016). Furthermore, *Lp* activates host defenses such as generation of ROS (Jones et al., 2013), protection from damaging agents (Jones et al., 2015), and defenses against pathogenic microbes (Blum et al., 2013). Remarkably, many host responses to *Lactobacilli* are conserved across vast evolutionary distances, as *Lactobacillus* strains coordinate nutrient acquisition (Schwarzer et al., 2016), ROS generation (Jones et al., 2013), and gut defenses in mouse models (Jones et al., 2015). These observations position the fly as a valuable model to examine developmental and homeostatic contributions of *Lactobacilli* to animal health (Matos and Leulier, 2014).

Our interest in *Lp* arose from an earlier study where we showed that elimination of the microbiome from adult flies extended their lifespan relative to conventionally-reared (CR) flies (Petkau et al., 2014). Based on this observation, we hypothesized that one or more species of commensal bacteria shortens the lifespan of adult *Drosophila*. In this study, we tested common *Drosophila* commensals for effects on adult lifespan. Of all species tested, we found that *Lp* recapitulated the microbiome-mediated truncation of adult lifespan. Upon closer examination, we observed a number of severe intestinal pathologies in gnotobiotic flies associated with *Lp*

that included extensive damage to the midgut epithelium, loss of epithelial barrier integrity, and death of midgut progenitors. Remarkably, we found that *imd* mutants were insensitive to the negative effects of mono-association with *Lp*, suggesting that *Lp* diminishes adult longevity in an *imd*-dependent manner. To our knowledge, ours are the first studies to identify a pathobiont of adult flies, and suggest a link between commensal *Lp* and innate immune responses in the control of host lifespan.

RESULTS

Mono-association with Lp *diminishes adult lifespan.*

We and others found that germ-free (GF) adult *Drosophila* outlive CR flies (Clark et al., 2015, Petkau et al., 2014). To identify the species responsible for diminished adult longevity, we inoculated GF, wild-type flies with one of three common *Drosophila* commensals. Specifically, we generated gnotobiotic flies that we mono-associated with lab-specific isolates of *Acetobacter pasteuranius (Ap), Lactobacillus brevis (Lb),* and *Lactobacillus plantarum (Lp)*. In each case, we worked with GF adults that we derived from CR larvae. This allowed us to examine microbial effects on adult health without compromising larval development. We then monitored longevity of the respective gnotobiotic flies to determine species-specific impacts on adult lifespan. As expected, GF flies significantly outlived CR flies (Fig. 1A, B). Among the mono-associated populations, we found that *Lb* and *Ap* had little to no effect on fly longevity (Fig. 1A, B). In both cases, the mono-associated populations had lifespans that closely mirrored GF flies. In contrast, mono-association with *Lp* significantly decreased adult lifespan (χ^2 = 32.30, $p<0.0001$) compared to CR flies, with a substantially diminished median survival of 20 days compared to 29 days for CR flies. These findings indicate that mono-association with *Lp* shortens the lifespan of adult *Drosophila*.

Abundance of Lp impacts adult fly lifespan

As CR flies significantly outlived *Lp* mono-associated flies (Fig. 1B), we hypothesized that additional commensal species partially dampen the detrimental effects of *Lp* on CR populations. To test this hypothesis, we compared the longevity of CR flies to gnotobiotic flies that we mono-associated with *Lp*, or gnotobiotic flies that we poly-associated with defined commensal species. For these experiments, we fed GF flies a culture of live *Lp* alone, or *Lp* mixed with equal amounts of *Ap* and *Lb*. In this assay, we noted a substantial increase in the

numbers of bacteria that associated with adult guts over time (Fig. 1C). These findings overlap with earlier data that intestinal colonization by commensal bacteria increases as flies age (Blum et al., 2013, Broderick et al., 2014). However, we noticed that intestinal colonization by *Lp* was several orders of magnitude higher in mono-associated flies than in poly-associated flies (Fig. 1D), suggesting that partner commensals limit intestinal colonization by *Lp*. As *Ap* colonizes the gut to similar extents as *Lp* (Fig. 1C), we asked if *Ap* attenuates the pathogenic effects of *Lp*. To determine the impact of *Ap* on *Lp* pathogenesis, we measured the lifespan of gnotobiotic adult *Drosophila* that we co-associated with differing rations of *Ap* and *Lp*. Here, we observed a clear relationship between *Ap*:*Lp* input ratios and adult lifespan - the greater the ratio of *Ap* to *Lp*, the longer the lifespan of co-associated flies (Fig. 1E, F). Together, these data argue that common fly commensals such as *Ap* limit intestinal colonization by *Lp* and mitigate against the pathogenesis of *Lp*.

Adult midguts mount a limited defensive response against Lp

At present, we know very little about adult intestinal responses to *Lp*. As our data point to detrimental effects of *Lp* mono-association for adult flies, we examined critical aspects of intestinal responses to microbial challenges in flies mono-associated with *Lp*. *Drosophila* adults typically respond to commensal or pathogenic bacteria with production of AMP via the IMD pathway, generation of ROS, and activation of homeostatic proliferation in ISCs (Buchon et al., 2014). We first examined the production of ROS and AMP in the guts of CR, GF and *Lp* mono-associated flies. For these assays, we quantified the intestinal expression of *dual oxidase* (*DUOX*), a source of gut ROS, and *diptericin* (*dpt*), a reporter of IMD pathway activity. Consistent with previous reports that commensals activate antibacterial responses in the adult gut (Buchon et al., 2009), we observed elevated expression of *DUOX* and *dpt* in CR guts compared to GF guts (Fig. S1A, B). In contrast, we found that *Lp* mono-associated flies

expressed *DUOX* and *dpt* at lower levels than CR flies (S1A, B). These data suggest that *Lp* mono-associated flies have a diminished antibacterial responses relative to CR flies.

We then asked if intestinal colonization by *Lp* stimulates a proliferative response to maintain tissue homeostasis. For this assay, we examined the EGF growth factor pathway, an integral component of proliferative responses to intestinal bacteria (Buchon et al., 2010). Specifically, we quantified expression of the EGF ligand *spitz* (*spi*) and the *spi*-activating endopeptidase *rhomboid* (*rho*). As expected, we detected higher levels of growth factor signaling in CR flies than in GF flies (Fig. S1C, D). In contrast, we found that *spi* and *rho* were expressed at lower levels in the guts of *Lp*-associated flies than in CR flies (Fig. S1C, D). In fact, we found that *spi* was expressed at significantly lower levels in the midguts of *Lp* mono-associated flies relative to GF flies after 15 days (Fig. S1C). Combined, these data suggest that the adult midgut response to *Lp* is phenotypically distinct to that observed in CR flies.

Lp gnotobiotic flies lack intestinal progenitor cell markers.

As our data suggest that adult midguts do not engage the EGF pathway after association with *Lp*, we examined the morphology of adult posterior midguts that we associated exclusively with *Lp.* To determine the influence of *Lp* on midgut morphology, we visualized the posterior midguts of CR, GF, *Lb* mono-associated and *Lp* mono-associated flies at days 2 and 15-post association. We used anti-Armadillo and anti-Prospero immunofluorescence stains in *esg[ts]>GFP* flies to visualize cell borders, enteroendocrince cells, and intestinal progenitor cells, respectively. We did not observe differences between the different treatment groups at the early time point (Fig. S2). In each case, the midgut contained evenly spaced nuclei, regular GFP positive progenitors, and neatly organized cell boundaries. As expected, older CR midguts showed signs of tissue dysplasia and disorganization compared to the midguts of GF flies (Fig. 2B, C). Interestingly, we found that flies mono-associated with the non-pathogenic commensal

Lb were phenotypically indistinguishable from CR flies (Fig 2B, D). In both cases, posterior midguts contained unevenly spaced nuclei, irregular cell borders, and widespread GFP fluorescence (Fig. 2B, D), hallmarks of the dysplasia typically observed in older, CR flies (Biteau et al., 2008). In contrast, *Lp* mono-associated flies appeared to maintain a remarkably well-organized midgut epithelium as they aged. Despite, the physiological burden of *Lp* mono-association, we observed regularly spaced nuclei, defined cell borders, and an even distribution of enteroendocrine cells at day 15 (Fig. 2E). However, in contrast to the apparently pristine organization of the epithelium, we noticed a striking absence of GFP positive progenitors throughout the midgut of flies that we mono-associated with *Lp* for 15 days (Fig. 2E). These data suggest an unexpected impact of *Lp* on the maintenance of progenitor cells in adult flies.

Impaired intestinal proliferation in Lp mono-associated flies.

The apparent loss of progenitor cells in *Lp* mono-associated flies prompted us to examine the proliferative capacity of ISCs in midguts associated with *Lp*. For this study, we used the MARCM clonal marking method to assess stem cell proliferation and subsequent differentiation in adult flies. MARCM allows investigators to identify mitotic cells and their progeny as GFP positive groups (Wu and Luo, 2006). As expected, GF flies had a limited number of small mitotic clones, whereas CR flies had more clones that contained greater numbers of cells (Fig. 2F, G). In contrast, we observed a near total absence of mitotic clones in the midguts of flies that we mono-associated with *Lp* (Fig. 2H). The few clones that observed invariably held fewer cells than age-matched clones in CR flies (Fig 2K). In addition, cells in clones from *Lp* mon-associated flies were morphologically distinct from those observed in CR flies (Fig. 2I, J). These results, in conjunction with our findings regarding impaired growth factor signaling and loss of progenitor cells, suggest that adult *Drosophila* fail to activate proliferative homeostatic repair mechanisms in response to the growing burden of *Lp*.

Lp disrupts posterior midgut ultrastructure.

To fully examine the effects of *Lp* on adult midgut ultrastructure, we used transmission electron microscopy to visualize the posterior midguts of age-matched CR and *Lp* mono-associated flies. Inspection of CR midguts revealed the anticipated sheath of visceral muscle that surrounds basal progenitor cells (Fig. 3A, B). Progenitors, in turn, support the generation of columnar epithelial cells that with an apical surface that faces the intestinal lumen (Fig. 3B, C). Upon examination of midguts associated with *Lp*, we were struck by substantial alterations to the intestinal epithelium. In CR flies, the epithelium consisted of continuous, regularly shaped cells with clearly visible nuclei and a flat apical surface (Fig. 3A–C). In contrast, the epithelium of *Lp* mono-associated flies contained an undulating population of cells (Fig. 3D, E) with large vacuoles (Fig. 3E, F) and poorly-discernible nuclei (Fig. 3D-F). We also noticed severe alterations to the morphology of presumptive progenitor cells. In place of the small, densely staining progenitors intimately associated with the visceral muscle of CR flies (Fig. 3B), mono-association with *Lp* led to the appearance of misshapen cells that did not associate properly with the muscle, had large, lightly staining nuclei, and contained numerous cytosolic vacuoles (Fig. 3D, E). Combined, these findings show that colonization of the adult midgut with *Lp* causes a pronounced intestinal pathology, characterized by thinning of the epithelium, formation of large cytosolic vacuoles, and a loss of progenitor cells. The extent of intestinal damage led us to determine the effects of *Lp* mono-association on midgut barrier integrity. For this experiment, we used the smurf assay, a convenient measure of intestinal barrier function (Rera et al., 2012). We found that mono-association of adult flies with *Lp* invariably caused a loss of gut barrier function immediately prior to adult death (Fig. 3G). In short, our data show that association of the adult midgut with *Lp* leads to a number of intestinal pathologies, loss of epithelial barrier function, and a premature death of the host.

Imd is essential for Lp-mediated pathogenesis.

In agreement with a recent study that showed partial activation of IMD responses by *Lp* in larvae (Erkosar et al., 2015), we noted a modest induction of *dpt* in *Lp* flies at day 15-post inoculation (Fig. S1). This prompted us to examine interactions between *imd*, *Lp* and host viability. To address this question, we determined the viability of CR, GF and *Lp* mono-associated *imd* mutants flies ($Imd^{EY08573}$). Similar to wild-type flies, we found that GF *imd* mutant flies outlived CR adults (Fig. 4A, B). However, in contrast to CR wild-type flies, we did not observe a negative impact of *Lp* on the viability of *imd* mutants. Whereas *Lp* shortened the lifespan of wild-type flies, we found that *imd* mutants mono-associated with *Lp* significantly outlived CR *imd* mutants (Fig. 4A, B). Indeed, *Lp* only moderately shortened the lifespans of *imd* mutants relative to GF *imd* mutants (Fig. 4B). Together, these data show that IMD is required for the negative effects of *Lp* on adult longevity.

DISCUSSSION

Conventional narratives partition intestinal microbes into convenient functional groups – pathogens that damage their host; benign passengers that exert little to no influence; or beneficial symbionts that equip their hosts with probiotic metabolites. The reality of host-microbe interactions is considerably more complex and requires an integrated appreciation of the environment, the microbiome, and the host genotype. Shifts in any one of these factors can drive dysbiosis among the commensal community and convert symbionts to pathogens (pathobionts) that fuel chronic diseases within their host (Kamada et al., 2013a). *Clostridium difficile* is a classic example of this paradigm: 2 – 5% of the North American population carry *C.* difficile, often without notable effects. However, simple changes such as antibiotic exposure can eradicate neighboring microbes and permit expanded colonization of the colon by *C. difficile*, leading to the development of colitis in afflicted individuals (Keller and Kuijper, 2015). In this study, we used *Drosophila melanogaster* as a tool to identify and phenotypically characterize a novel fly pathobiont, *Lactobacillus plantarum*. *Lactobacilli* are widely reported fly and mammal commensals with numerous established benefits for their animal hosts (Walter, 2008).

To our knowledge, our data are the first to show a detrimental impact of a common *Drosophila* commensal bacteria on the maintenance or proliferation of ISCs. We found that mono-association with *Lp* impairs ISCs proliferation, damages the intestinal epithelium, causes a breakdown of gut barrier function, and shortens adult lifespan. A previous study showed that *Gluconbacter morbifer,* causes disease in adult *Drosophila* if allowed to expand within the host (Ryu et al., 2008). However, *G. morbifer* is a comparatively rare fly commensal, and disease onset requires impaired immunity within the host. In contrast, this report identify a novel disease phenotype associated with unchecked expansion of a common fly commensal in a wild-type

host. We believe these findings represent a valuable model to define the mechanistic basis for host damage by a pathobiont.

As *imd* mutants appear relatively indifferent to mono-association with *Lp,* it appears the innate immune IMD response is critical for *Lp*-dependent pathogenesis in the fly. It is attractive to assume that this phenotype arises from collateral damage through deregulated expression of toxic immune effectors such as AMP or ROS. However, we believe it unlikely that IMD-responsive bactericidal effectors are responsible for the negative effects of *Lp* on host lifespan, as CR flies outlive *Lp* mono-associated flies despite increased AMP and DUOX expression. In this context, we consider it important to consider that several transcriptional studies demonstrated that a relatively small fraction of IMD-responsive transcripts are easily categorized as bacteriostatic or immune modulatory (De Gregorio et al., 2002). In fact, it seems that intestinal IMD activity primarily modifies metabolic gene expression (Broderick et al., 2014, Erkosar et al., 2014). As intestinal microbes are known to control nutrition and metabolism in their *Drosophila* host (Wong et al., 2014), we consider it possible that the *Lp*-dependent pathologies described in this study reflect an underlying imbalance in IMD-dependent regulation of host metabolism.

Consistent with possible links between *Lp*, IMD and host metabolism, it is noteworthy that a recent study established a direct link between *Lp* and the IMD-dependent expression of intestinal peptidases (Erkosar et al., 2015). Our data show that intestinal colonization by *Lp* is much greater in mono-associated flies than in poly-associated flies. We speculate that the elevated levels of *Lp*, combined with the absence of additional commensal, alters metabolic responses in the host, leading to impaired intestinal function. This hypothesis includes the possibility that *Lp* directly affects host diet, as proposed for other *Drosophila*-associated microbes (Chaston et al., 2014, Yamada et al., 2015). We are currently testing this hypothesis in flies with modified intestinal IMD activity.

Our work was initially inspired by reports from our group and others that GF adults outlive CR flies (Clark et al., 2015, Petkau et al., 2014). However, other studies reported variable impacts of the effects of microbiome removal on adult lifespan (Brummel et al., 2004, Ren et al., 2007). We believe that the differences between the individual reports reflects the intricate nature of interactions within a host-microbe-environment triad. For example, research groups typically raise their flies on an incompletely defined, oligdic diet that exerts underappreciated influences on the metabolic outputs of intestinal bacteria, and the transcriptional outputs of the host. We believe that a complete evaluation of the relationship between microbes and their hosts requires consideration of environmental inputs such as diet. Likewise, it is important to consider genotypic inputs of the symbiont and the host. For example, the beneficial contributions of *Lp* to mouse and larval nutrition display strain-specific effects (Schwarzer et al., 2016), suggesting variable effects of individual *Lp* strains on host phenotypes. In addition, most research groups study lab-raised strains of experimental models, a situation that allows unexplored genetic drift within the host to influence microbiome composition and phenotype.

In summary, this report identifies *Lp* as a novel pathobiont of adult flies. To our knowledge, ours is the first study to report such a class of microbe in flies. Given the negative effects of pathobionts on animal health, and the experimental accessibility of *Drosophila* and *Lactobacilli*, we believe these findings represent a valuable tool for the definition of the mechanisms by which shifts in symbiotic populations lead to pathologies in tolerant hosts.

EXPERIMENTAL PROCEDURES

Fly husbandry

All experiments were performed at 29°C with virgin female w^{1118} flies. Additional genotypes are provided in supplemental material. Flies were raised on standard corn meal medium (Nutri-Fly Bloomington Formulation, Genesse Scientific). Germ-Free flies were generated by raising adult flies on autoclaved standard media supplemented with an antibiotic solution (supplemental material). For longevity studies, flies were raised in vials with 10 flies per vial, and transferred to fresh vials weekly. Colony forming units per fly were determined by independent quantification of 4 replicates with 5 flies/replicate. Flies were sterilized in 50% bleach, 75% ethanol and rinsed in water. Sterilized flies were homogenized in MRS broth (Fluka Analytical) and serial dilutions of homogenate were plated on MRS to identify *Lactobacillus* species and GYC agar plates to identify *Acetobacter* species. Lactobacillus species were distinguished by colony morphology.

Generation of gnotobiotic *Drosophila*

Virgin females were raised on antibiotic supplemented medium for 5 days at 29°C. Flies were starved in sterile empty vials for 2 hours prior to bacterial association. Lab isolate *Ap* was grown in Mannitol broth at 29°C with shaking 2 days prior to association. Lab isolates *Lb* and *Lp* were grown in MRS broth at 29°C 1 day prior to association. For mono-associations, bacterial cultures were re-suspended in 5% sucrose/PBS to an OD_{600} of 50. For co-associations, bacterial cultures of *Ap* and *Lp* were prepared to an OD_{600} of 50 each in 5% sucrose/PBS. The bacterial cultures were then mixed at the selected ratios. For poly-associations, bacterial cultures of *Ap, Lb,* and *Lp* were prepared to an OD_{600} of 50 in 5% sucrose/PBS, and mixed at a 1:1:1 ratio. For all bacterial associations, 12 flies/vial were fed 1ml bacterial suspension on autoclaved cotton plugs for 16 hours at 29°C. To ensure maintenance of mono-association or

GF conditions, respective flies were homogenized in MRS broth and plated on MRS or GYC agar plates periodically (1-1.5 weeks) throughout the study.

Immunofluorescence

Flies were washed with 95% ethanol and dissected in PBS to isolate adult intestines. The complete immunofluorescence protocol is provided in the supplementary material. The primary antibodies used in this study were as follows: mouse anti-Armadillo (1:100; DSHB N2 7A1), mouse anti-Prospero (1:100; DSHB MR1A). Guts were mounted on slides in Fluoromount (Sigma-Aldrich F4680). The posterior midgut was visualized with a spinning disk confocal microscope (Quorum WaveFX, Quorum Technologies Inc.). Images were collected as z-slices and processed with Fiji software to generate a single *Z*-stacked image. For transmission electron microscopy, posterior midguts were excised and immediately placed into fixative. Fixative preparation, sectioning, staining and visualization were performed at the Faculty of Medicine and Dentistry Imaging Core at the University of Alberta.

qPCR

Realtime PCR was performed on the dissected guts of adult *Drosophila* as described previously (Guntermann and Foley, 2011). Sequences of the individual primers is provided in the supplementary materials as methods.

AUTHOR CONTRIBUTIONS

D.F and E.F. conceived and designed experiments; D.F and A.D. performed the experiments; D.F. and E.F performed data analysis and wrote the paper.

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Figure Legends

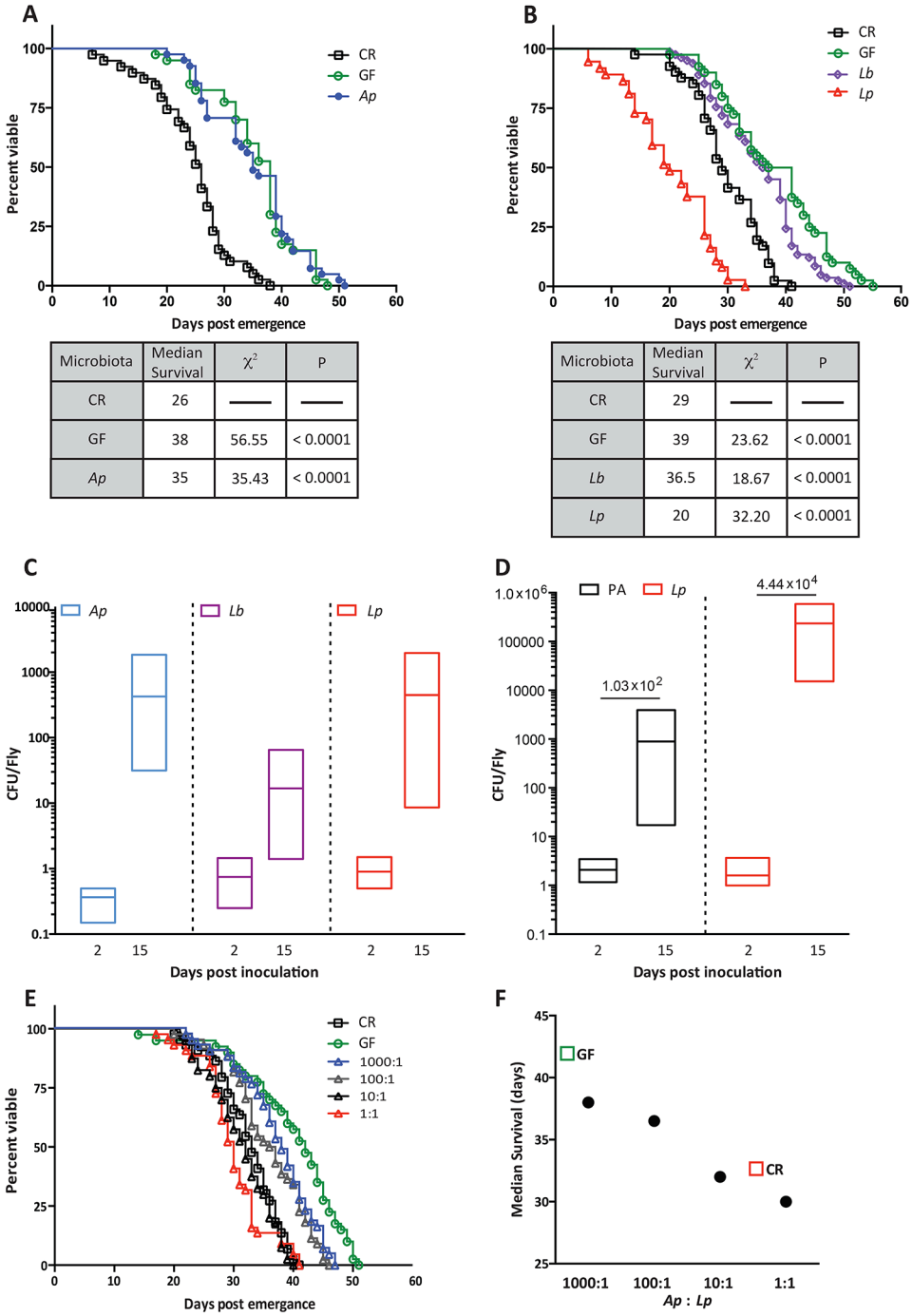
Figure 1. Mono-association with *Lp* reduces adult fly lifespan. (A, B). Survival curves of CR, GF, *Ap, Lb, Lp* gnotobiotic virgin females (50 flies/group). Y-axis shows percent survival and x-axis shows time after emergence in days. χ^2 and p values are relative to CR flies. Tables show Log-rank (Mantel-Cox) test of panel A and B. **(C)** Colony forming units (CFU) per fly of *Ap*, *Lb*, and *Lp* in poly-associated (PA) flies. **(D)** CFU per fly of *L. plantarum* in PA and *Lp* mono-associated flies. Numbers in black indicate fold change in the means at day 15 relative to day 2. **(E)** Survival curves of CR, GF, and *Ap/Lp* flies co-associated at indicated ratios (50 flies/group). Tables show Log-rank (Mantel-Cox) tests. χ^2 and p values are relative to CR flies. **(F)** Median survival of data shown in panel E.

Figure 2. *Lp* blocks ISC proliferation. (A) Schematic representation of the generation of mono-associated *Drosophila.* **(B-E)** Immunofluorescence of posterior midguts of CR **(B)**, GF **(C)**, *Lb* mono-associated **(D)**, or *Lp* mono-associated flies **(E)** at day 15 post inoculation. Guts were stained with Hoechst and anti-Arm/Pros antibodies as indicated. Progenitor cells were visualized with GFP as indicated. Hoechst (blue), GFP (green), and anti-Arm/Pros (red) were merged in the fourth row. Scale bars are 25 µm. **(F-J)** GFP-positive MARCM clones from the posterior midgut of CR **(F)**, GF **(G)** and *Lp* mono-associated flies **(H)** at day 15-post inoculation. Hoechst (blue) and GFP (green) were merged in the third row. Scale bars are 25 µm. **(I,J)** Higher resolution of clonesfrom CR and *Lp* flies boxed in F and H, respectively. Scale bars are 5 µm. **(K)** Quantification of cells/clones in CR, GF and *Lp* flies.

Figure 3. *Lp* disrupts posterior midgut ultrastructure. (A-F) Transmission electron microscopy of CR and *Lp* mono-associated posterior midguts 15 days after inoculation. Epithelial cells (E),

progenitors (P), progenitor pairs (PP), and visceral muscle (VM) are labeled. **(A, D)** 1200x direct magnification. Scale bars are 5 µm. **(B, E)** 3000x direct magnification. Scale bars are 1µm. **(C, F)** 3500x direct magnification. Scale bars are 1µm. Black arrow heads indicate vacuoles. **(G)** Intestinal integrity assay (Smurf) of *L. plantatrum* mono-associated flies.

Figure 4. *Lp* pathogenesis involves IMD activity. (A) Survival curve of CR, GF, and *Lp* mono-associated $Imd^{EY08573}$ virgin female flies (100 flies/group). Table show Log-rank (Mantel-Cox) test for data in A. χ^2 and p values are relative to CR flies. **(B)** Quantification of fly death in CR, GF and *Lp* gnotobiotic w^{1118} and $Imd^{EY08573}$ flies.



Microbiota	Median Survival	χ^2	P
CR	26	—	—
GF	38	56.55	< 0.0001
Ap	35	35.43	< 0.0001

Microbiota	Median Survival	χ^2	P
CR	29	—	—
GF	39	23.62	< 0.0001
Lb	36.5	18.67	< 0.0001
Lp	20	32.20	< 0.0001

Microbiota	CR	GF	1000:1	100:1	10:1	1:1
Median Survival	33	42	36.5	35	32	30
χ^2	—	33.51	20.27	12.40	1.129	2.849
P	—	< 0.0001	< 0.0001	0.0004	0.2880	0.0914

Figure 1.

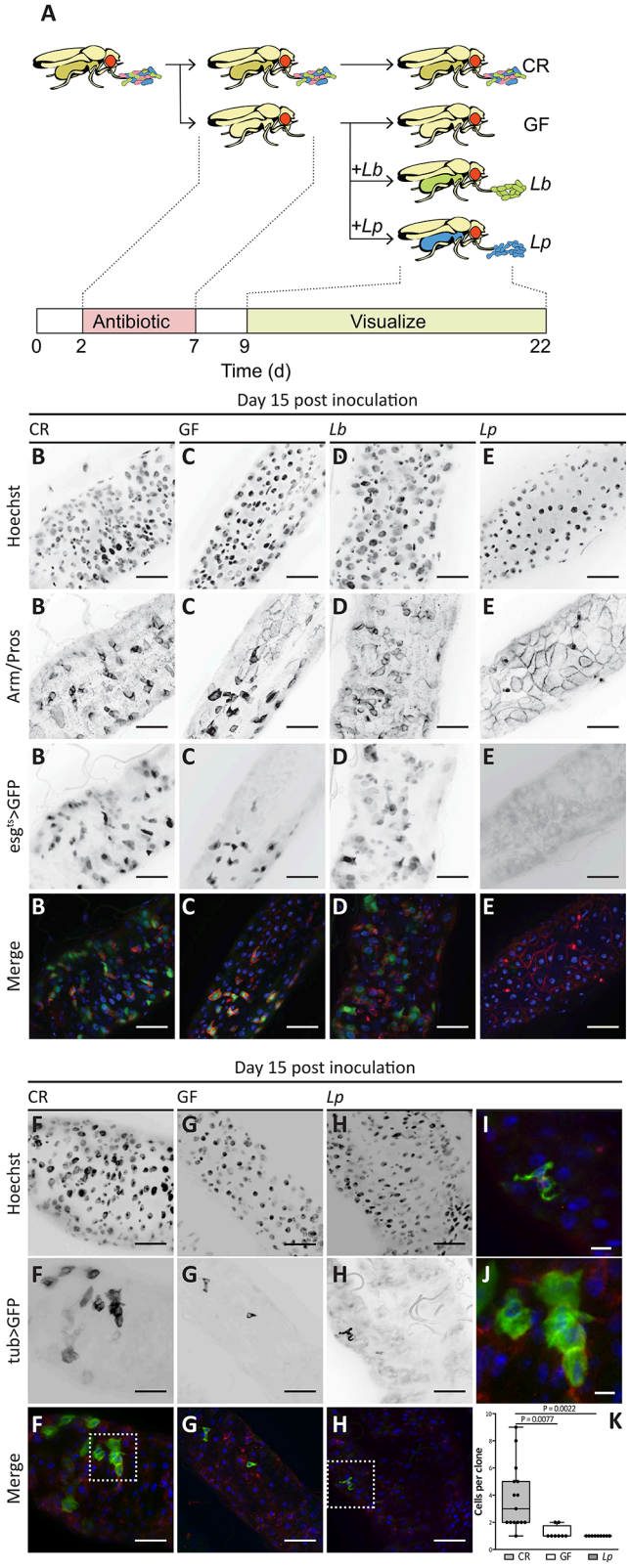
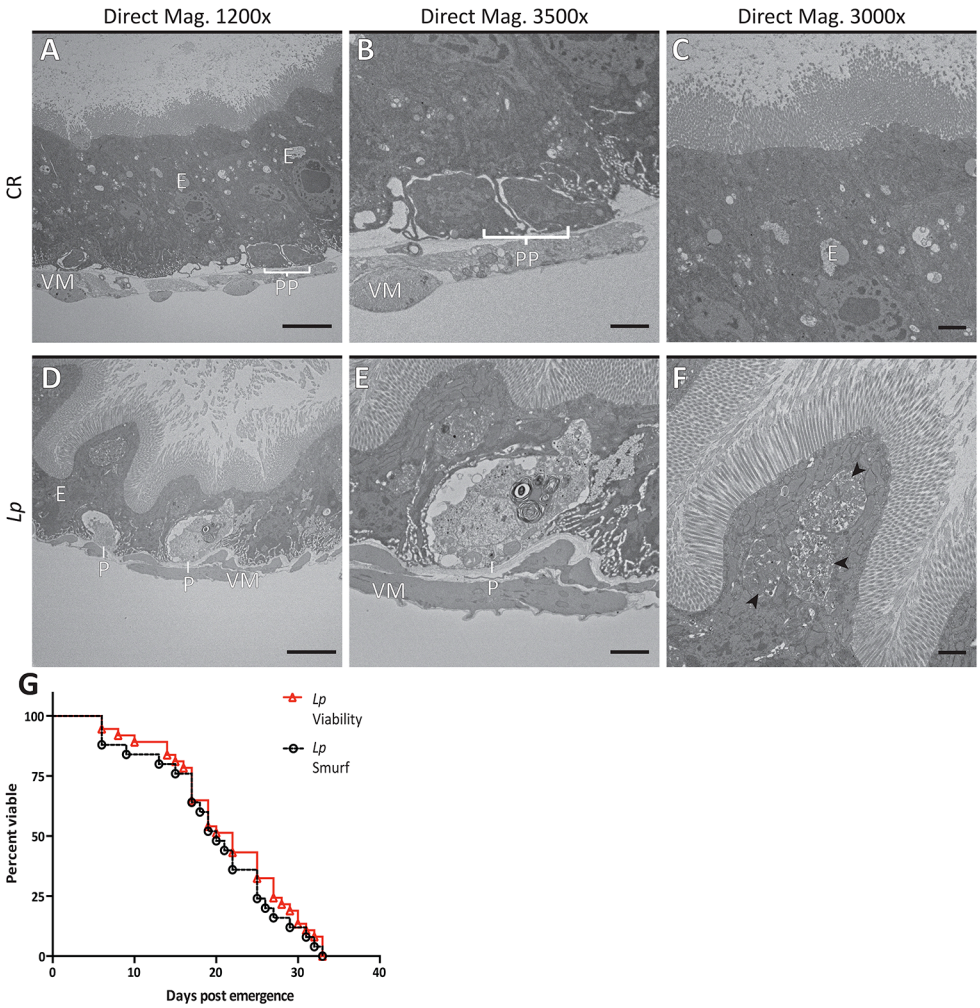

A
CR
GF
+Lb
Lb
+Lp
Lp
Antibiotic
Visualize
0
2
7
9
22
Time (d)
Day 15 post inoculation
CR
GF
Lb
Lp
Hoechst
Arm/Pros
esg^ts>GFP
Merge
B
C
D
E
Day 15 post inoculation
CR
GF
Lp
Hoechst
tub>GFP
Merge
F
G
H
I
J
K
P = 0.0022
P = 0.0077
Cells per clone
CR
GF
Lp

Figure 2.


Direct Mag. 1200x
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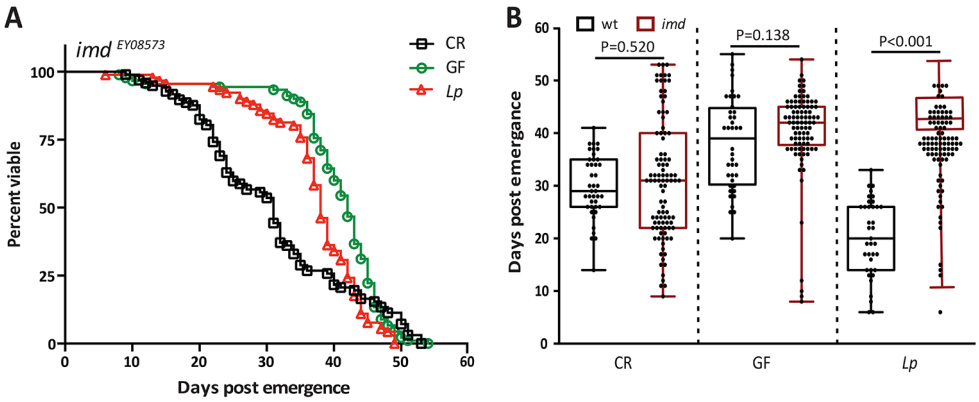
CR
Lp
A
B
C
D
E
F
E
VM
PP
P
G
Lp Viability
Lp Smurf
Percent viable
Days post emergence
0
25
50
75
100
10
20
30
40

Figure 3.



Microbiota	Median Survival	χ^2	P
CR	31	—	—
GF	42	10.92	0.0010
Lp	38	2.279	0.1312

Figure 4.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Fly stocks.

The *w, esg-GAL4, tubGAL80[ts], UASGFP* and *imd[EY08573],* flies have been described previously (Avadhanula et al., 2009, Buchon et al., 2010). Mitotic clones were generated with flies of the genotype *y,w, hs-flp, UAS-mCD8GFP; neoFRT(40A)/neoFRT(40A),tubGAL80; tubGAL4/+*. The antibiotic solution (100 µg/ml Ampicillin, 100 µg/ml Metronidazole, 40 µg/ml Vancomycin dissolved in 50% ethanol and 100 µg/ml Neomycin) used in this study has been described elsewhere (Ryu et al., 2008).

Immunofluorescence

Guts were fixed for 20 minutes at room temperature in 5% formaldehyde in PBS. Guts were rinsed in PBS for 20 minutes at room temperature and blocked overnight in PBSTBN (PBS, 0.05% Tween 20, 5% BSA, and 1% goat serum) at 4°C. Guts were stained overnight at 4°C in PBSTBN with appropriate antibodies, washed with PBSTB (PBS, 0.05% Tween 20, and 5% BSA) and stained for 1 h at room temperature in PBSTBN with Hoechst 33258 (1:500) and the appropriate secondary antibody (goat anti-mouse Alexa Fluor 568) guts were washed with PBSTB and rinsed with PBS prior to visualization.

SUPPLEMENTAL REFERENCES

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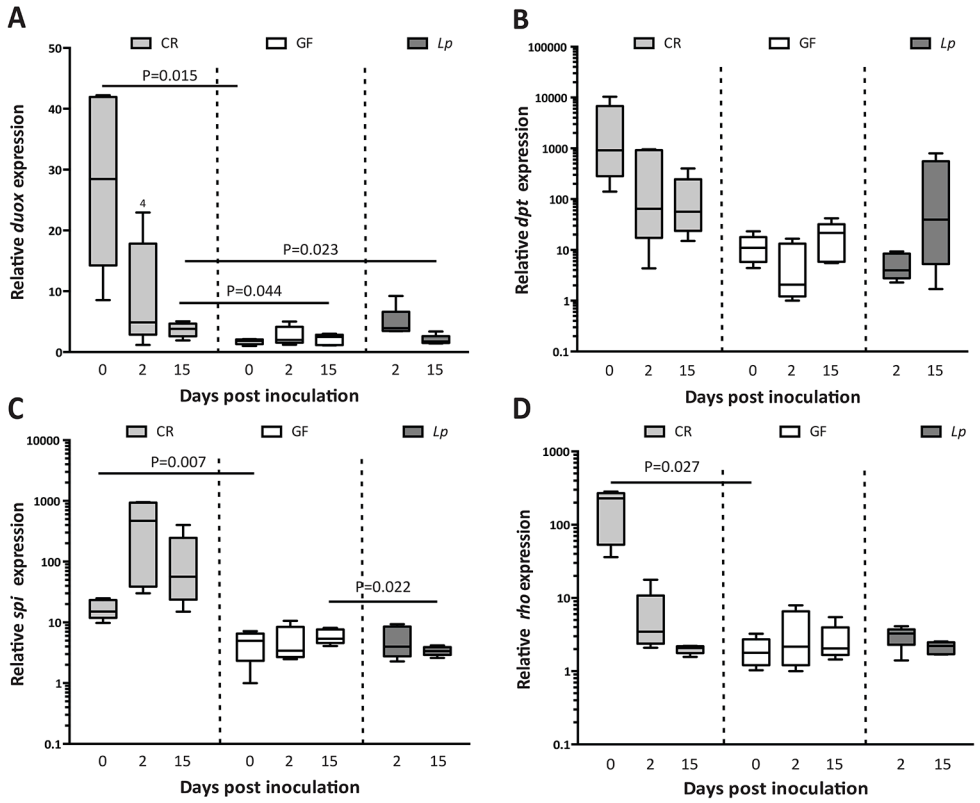
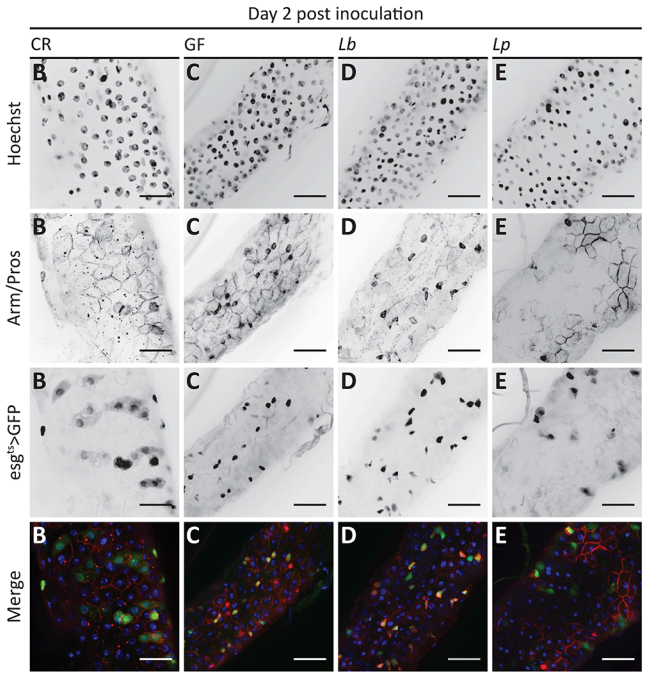


Supplementary Figure 1: Quantitative real-time PCR analysis of expression of gut immunity proteins of *DUOX* **(A),** *diptericin* **(B)** growth factor cytokine *spitz* **(C)** and endopeptidase *rhomboid* **(D)** from the dissected guts of adult CR, GF, and *Lp* gnotobiotic flies. Each time point represents five independent measurements. 4 Denotes times points of 4 measurements. Expression levels were standardized to actin in the respective groups.



Supplementary Figure 2: Immunofluorescence of posterior midguts of CR (first column), GF (second column), *Lb* mono-associated (third column), or *Lp* mono-associated flies (fourth column) 2 days after inoculation. Guts are stained with Hoechst (first row) and anti-Arm/Pros antibodies (second row). Intestinal progenitor cells were visualized with *esg[ts]* > GFP (third row). Hoechst (blue), GFP (green), and anti-Arm/Pros (red) were pseudo-colored and merged in the fourth row. All scale bars are 25 µm.