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Alcaraz, Jeanette

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Microbiota and immune contributions to age-related intestinal decline

A thesis submitted in partial satisfaction
of the requirements for the degree
Master of Science in Physiological Science

by

Jeanette Alcaraz

2016

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ABSTRACT OF THE THESIS

Microbiota and immune contributions to age-related intestinal decline

by

Jeanette Alcaraz

Master of Science in Physiological Science

University of California, Los Angeles, 2016

Professor David William Walker, Chair

Human microbiota encompass the populations of microorganisms that live on and in human organ systems. Importantly, a large proportion of the microbiota lies in the digestive tract. Gut microbes facilitate beneficial, and necessary, metabolic and protective processes within their hosts. Imbalances in gut microbial load or composition may lead to inflammation and changes in intestinal physiology, ultimately impacting whole organism health. Currently, we understand that age-related changes in gut microbiota are a primary source of immune activation. However, the cause-effect relationships between microbiota dynamics, immune activity, and changes in intestinal health are not yet fully understood. The fruit fly, *Drosophila melanogaster*, has enabled us to show how chronic immune activation in young flies drives early-onset mortality as well as increased loss of barrier function. Changes in digestive transit and microbial populations are also characteristics of flies that experience immune activation throughout life. Thus, there are clear physiological changes that occur not just with age, but also as a product of immune responses in the fly. A better understanding of

microbial and immune pathway dynamics may allow for development of interventions to delay age-related decline.

The thesis of Jeanette Alcaraz is approved.

Scott H. Chandler

Mark Arthur Frye

Rebecca Clark

David William Walker, Committee Chair

University of California, Los Angeles

2016

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Introduction

Human microbiota encompass the populations of microorganisms that live on and in human organ systems. Importantly, a large proportion of the microbiota lies in the digestive tract. There are a number of factors that can influence gut microbiota composition in mammalian systems (Bischoff, 2016, Claesson et al., 2012). For example, mild or extended use of antibiotics can drastically change microbial populations in the gut. Importantly, aging is also a physiological process that changes bacterial load and composition in the gut (Clark et al., 2015, Guo et al., 2014). Both natural and lifestyle related changes in microbial composition are critically important. Gut microbes facilitate beneficial, and necessary, metabolic and protective processes within their hosts (Sommer et al., 2013). Thus, regulation of microbial populations is highly important in maintaining organismal homeostasis. Imbalances in gut microbial load or composition may lead to inflammation and changes in intestinal physiology, ultimately impacting whole organism health.

Although a number of recent publications have shown shifts in intestinal microbiota populations with age, these studies are mostly limited to correlative data (Claesson, 2011, Biagi et al., 2010). Two major questions that remain unanswered are as follows: Are changes in microbiota dynamics linked to distinct events in the pathophysiology of aging? What are the cause-effect relationships between microbiota dynamics, immune activity, and changes in intestinal health? My project aims to better understand how immune activity may be affecting age-related changes in gut microbiota. More broadly, I am interested in understanding how this relationship may effect overall intestinal physiology and organismal health. As a model organism,

Drosophila melanogaster has a relatively short lifespan and diverse capacity for genetic manipulation. Additionally, the fly intestine hosts a simple microbial population, that nonetheless contains a number of taxa also found in the human intestine (Broderick, 2016). This makes the fruit fly ideal for studying longevity and physiological changes that occur with age. A better understanding of microbial and immune pathway dynamics, developed in the *Drosophila* model, may allow for further interventions to delay age-related decline.

In an analogous fashion to humans there are also detrimental physiological changes that occur in the fly with age. One of many age-related changes in the fly is intestinal barrier dysfunction. Upon loss of intestinal integrity, flies exhibit a leaky gut phenotype where the entire fly turns blue upon being placed on blue-dyed food. These blue flies are termed Smurfs and will be called such hereafter. Interestingly, the proportion of Smurf flies in any given fly population is closely correlated with age. With time the aging fly population will exhibit an increased proportion of flies with loss of barrier function. Previous work has shown that Smurf proportions substantially increase at median survival. Additionally, control or non-Smurf flies have longer lifespans (Rera et al., 2012). Taken together, these results indicate that loss of barrier function is an age-related pathology that decreases fly survivorship.

Inflammation is another hallmark of aging both in the fly and in mammalian systems. Past work in flies has shown increases in immunity gene expression. These changes occurred in the individual fly and at the population level. Particularly, flies suffering from intestinal barrier dysfunction (Smurfs) show individual increases in immune activity. Two immune pathways, the Toll and Immune Deficiency (IMD)

pathway, were the focus of past work and are the focus of my thesis project. These pathways are subject to activation in the whole fly through a tissue called the fat body; additionally these pathways can be locally activated at the level of the fly intestine. Intestinal epithelial cells contain receptors on the plasma membrane, which activate the Toll and IMD pathways respectively. These immune pathways are regulated independently, but can also act synergistically (Tanji et al., 2007). The Toll membrane receptor, *Toll*, is sensitive to gram-positive bacteria and fungi. The IMD membrane receptor, *PGRP-LC*, is sensitive to gram-negative bacteria. Upon receptor activation a cascade of intracellular events is induced, ending in activation of a nuclear transcription factor (Valanne et al., 2011). This transcription factor will induce transcription of specific antimicrobial peptides (AMPs), which will then participate in a number of mechanisms aimed to protect the host (Buchon et al., 2009b). AMP expression may then be used as a marker for immune activity. Even with an understanding of immune pathway mechanisms, the question that remains is, how does immune activation relate to overall organism health? More specifically, what effects if any do immune activities have on microbiota and age-related decline?

My preliminary data shows that immune activation in young flies drives early-onset mortality as well as increasing loss of intestinal barrier function. Changes in digestive transit are also characteristic of flies that experience chronic immune activation throughout life. Interestingly, inducing immune activity in young flies also drives changes in microbial populations. We've come to understand that age-related changes in gut microbiota are a primary source of immune activation (Rera et al., 2012, Clark et al., 2015). Thus, there are clear physiological changes, which occur not just

with age, but also as a product of immune responses in the fly. Although these changes have been detected, we still need to distinguish between immune and microbial impacts on intestinal function.

Materials and Methods

Fly culture and Lifespan

Genotypes used to conduct experiments include laboratory strain *w*¹¹¹⁸, 5966 GeneSwitch and UAS-Toll10^b, UAS-PGRP-LC, UAS-PGRP-LC-IR, and UAS-Rel-IR. Flies were housed in vials with a population density of approximately 30 flies per vial. Vials were kept in a 25°C environmental room equip with 12 hour on/off light cycle. All fly vials were flipped onto new medium every Monday, Wednesday, and Friday. On these days, flies were scored as dead or alive if they were being used for longevity assays. During experimental sorting (e.g. male from female, Smurf from non-Smurf, etc.) adult flies were anesthetized using low levels of nitrogen gas.

Medium Preparations

Standard medium was prepared by mixing 1%(wt/vol) agar, 3% (wt/vol) yeast, 1.9% (wt/vol) sucrose, 3.8% (wt/vol) dextrose, and 9.1% (wt/vol) cornmeal. Blue medium was prepared by adding blue dye no.1 at a concentration of 2.5% (wt/vol) to standard medium. Drug-induction medium, or RU medium, was prepared by mixing RU-486 at a dose of 50µg/ml with standard medium. RU-486 (Cayman Chemical Company) was first dissolved in ethanol prior to medium prep. RU control medium contained an equivalent volume of ethanol only. Bromophenol-blue medium was prepared by mixing 0.5% (wt/vol) Bromophenol blue sodium salt with standard medium.

Smurf Assay

Flies were placed on blue medium for a period of 24 hours prior to Smurf scoring. At the 24th hour, flies were scored as Smurfs when the blue dye was visible anywhere outside

of the digestive tract. Post-scoring, flies were flipped back onto standard or experiment (RU) media.

Fecal Plate Preparations

Flies were first placed on blue medium for 24 hours to be sorted as Smurf versus non-Smurf. Non-Smurf flies were then placed into vials containing Bromophenol-blue medium for 24 hours to ensure all flies would excrete pH sensitive medium. Flies involved in fecal spot analysis were placed into small groups based on condition. There were 10 total plates per condition. Each plate contained 10 flies for a total of 100 flies per condition. Flies were allowed to excrete onto petri dish surfaces (lid and bottom) for 24 hours. Bromophenol-blue medium was offered as a wedge placed within the petri dish for the duration of the experiment. At the end of the experiment, flies and medium were removed from the plates and a high-resolution image was obtained with an Epson Perfection V200 scanner. Images were later cropped with FIJI imaging software and analyzed with The Ultimate Reader of Dung (T.U.R.D).

Quantitative PCR

Flies were placed on blue medium for 24 hours prior to bacterial sample collection. Whole-fly bacterial samples were collected after flies had been sorted as Smurf or non-Smurf. Only non-Smurfs were collected and at each respective time point. The PowerSoil DNA Isolation Kit (MoBio) was used to extract DNA from whole-fly bacterial samples. Prior to following manufacture's DNA isolation instructions, flies were first surface sterilized as previously described (Ren et al., 2007) before further sample preparation. Samples were then homogenized in 150 μ L of PowerSoil bead tube solution using a motor pestle. Homogenate was then returned to bead tube and the remainder of

the protocol was followed. Bacterial DNA samples were amplified to discern bacterial load changes via drug-induced immune activation. PCR was performed with *Power SYBR Green* master mix (Applied Biosystems) on an Applied Biosystems 7300 Real Time PCR system. Quantitative PCR were run with universal and taxon specific primers targeting bacterial 16S, a ribosomal RNA gene, and fly actin, as a loading control. All primer sequences used are as previously described (Clark et al. 2015).

Statistical Analysis

Survival curve analysis was completed using the log-rank test in Graphpad Prism software. To determine whether the Smurf proportion between the drug-induced and uninduced control groups was significantly different a binomial distribution test was run using R statistical software (v 3.1.2). Prepared fecal images were analyzed via image analysis software called The Ultimate Reader of Dung or abbreviated as T.U.R.D. Default settings were changed in T.U.R.D to minimize fecal plate artifact and optimize fecal spot annotations: Block Size = 65, Offset = 8, Minimum = 25, Maximum = 1000, Brush Size = 1. Statistical significance was computed using the Mann-Whitney U rank test in T.U.R.D and cross referenced with a secondary built-in rank test in R. To compare bacterial amplification across conditions and between time-points, qPCR target gene 16S was normalized to actin control. Significant differences between time-points were analyzed using a two-sided Wilcoxon Test in R.

Results

Activation of Immune Pathways Leads to Increased Barrier Dysfunction and Increased Mortality

Overexpression of the Toll and IMD pathway was achieved through overexpression of the respective membrane receptors, *Toll* and *PGRP-LC*. These particular genes were over expressed in the intestinal epithelium only via the Geneswitch-UAS transgene expression system. The 5966-driver line facilitated expression of a transcriptional geneswitch in the gut alone. Further, this geneswitch protein was only activated in the presence of steroid hormone, RU-486. The UAS portion of the transgene (*UAS-Toll10b*, *UAS-PGRP-LC*) bound to the activated geneswitch-steroid complex. Thus, transgene expression was controlled both temporally and spatially through application of a steroid ligand (Osterwalder et al., 2001).

Drug-induced activation of the Toll pathway inside fly gut epithelia preceded an increase in population mortality. Beginning at 20 days of age, flies began to show a steady decline in lifespan. Median survival, or 50% of the proportion of flies surviving, was 33 days for drug-induced flies versus 54 days in uninduced control flies. Overall, activation of the Toll pathway in flies was correlated with an 18-day decrease in lifespan or 26.7% overall decrease in maximum survival. The final age of drug-induced flies was 52 days, compared to 70 days for the uninduced control flies (Figure 1A). Coupled to an increase in mortality, at 20 and 30 days of age there was a significant increase in the proportion of flies having lost intestinal barrier function (Figure 1B).

Drug-induced activation of the IMD pathway preceded an increase in fly mortality. Beginning at 30 days of age, flies began to show a decline in lifespan. Median survival, or 50% of the proportion of flies surviving, was 36 days for drug-induced flies versus 66 days in uninduced control flies. Overall, activation of the IMD pathway in flies was correlated with a 34-day decrease in lifespan or 41.9% overall decrease in maximum survival. The final age of drug-induced flies was 47 days, compared to 81 days for the uninduced control flies (Figure 1C). Coupled to an increase in mortality, at 20 and 30 days of age there was an increase in the proportion of flies having lost intestinal barrier function (Figure 1D).

Immune Activation Impairs Digestive Transit

By feeding drug-induced and uninduced control flies pH sensitive medium, information regarding acid-base dynamics in the gut was obtained. Additionally, as food traveled through the fly digestive tract and was subsequently excreted, any fecal differences between groups of flies could be characterized and used as an indicator of intestinal health. The following results were characterized and obtained: mean area of individual fecal deposits, number of deposits, mean hue of deposits, and mean lightness of fecal deposits. Mean hue results provide information regarding the relative acid-base balance of intestinal contents. Thus, an increase in hue corresponds with a higher or more alkaline pH. By contrast, a decrease in hue corresponds with a decrease in pH. Mean lightness is used as a measure of fecal concentration or water reabsorption. It is quantified on a 0-1 scale, with increased lightness closer to 1 and decreased lightness, or more concentrated fecal spots, quantified closer to 0.

Drug-induced Toll activation resulted in a significant decrease in number of fecal deposits when compared to uninduced control samples (Figure 2E). Toll pathway activation also correlated with an increase in mean hue (Figure 2F). Regarding fecal concentrations, there was an observed decrease in lightness in drug-induced flies. Thus, Toll activation resulted in more concentrated fecal deposits (Figure 2G). Correlated with a decrease in lightness, or increase in fecal concentration, there is a significant decrease in mean area of the fecal deposits (Figure 2H). These results were obtained from 30 day old, non-Smurf flies.

Drug-induced activation of the IMD pathway was correlated with a significant decrease in total number of fecal deposits (Figure 2E). IMD activation was also correlated with an increase in hue (Figure 2F). Regarding fecal concentrations, there was an observed increase in lightness in drug-induced flies. Thus, IMD activation resulted in less concentrated fecal deposits (Figure 2G). Correlated with an increase in lightness, or decrease in fecal concentration, was a significant increase in mean area of the fecal deposits (Figure 2H).

Immune Activation Changes Whole Fly Bacterial Load and Composition

In addition to changes in mortality, intestinal barrier function, and intestinal physiology, immune activation of the Toll and IMD pathways also preceded changes in bacterial load and composition. Drug-induced Toll pathway activation in gut epithelia resulted in a 200-fold increase in bacterial load at 20 days of age. There was also a significant increase in bacterial load in drug-induced flies relative to uninduced control flies at 30 days of age (Figure 3A). Results regarding changes in bacterial compositions are shown for three specific classes of bacteria, which can be broadly categorized as

either gram-negative or gram-positive: Bacilli (gram-positive bacteria), Gammaproteobacteria (gram-negative bacteria), Alphaproteobacteria (gram-negative bacteria). Drug-induced activation of the Toll pathway did not significantly change levels of Bacilli or Gammaproteobacteria at 20 or 30 days of age (Figure 3C,D). Drug-induced flies did, however, show a significant increase in Alphaproteobacteria at 20 days of age, but not at day 30 (Figure 3E).

Activation of the IMD pathway resulted in a significant increase in bacterial load at 20 and 30 days of age (Figure 3B). Bacilli levels at 20 days of age were not significantly changed upon IMD activation. They were however, significantly increased at 30 days of age (Figure 3F). Gammaproteobacteria levels did not significantly change at 20 or 30 days of age (Figure 3G). Relative to uninduced control flies, Alphaproteobacteria levels significantly increased in drug-induced flies at 20 and 30 days of age (Figure 3F).

Discussion

In humans changes in intestinal physiology are typically associated with underlying pathologies such as Crohn's Disease or Irritable Bowl Syndrome (IBS). Incidence of IBS, as well as other conditions and disorders, increase with age (Lanzoni, 2008). Age-related inflammatory disorders are attributed to changes in microbiota and overall immunosenescence (Claesson et al., 2012). Presently, there are medications and surgical options for individuals suffering from intestinal disorders. These options, however, are invasive and/or have a number of deterring side effects. Through better understanding the mechanism underlying age-related intestinal decline, we may be able to better assist by treating the cause of these ailments instead of the symptoms.

Intestinal immune activation via the Toll and IMD pathways was shown to decrease barrier function and perturb intestinal physiology. Both of these changes preceded an increase in fly mortality when compared to uninduced control flies. Although most results were similar between Toll and IMD upregulation, there were some stark differences that may give further insight into the pathophysiology of the aging fly. After only 20 days of drug-induction, IMD activation was correlated with a striking increase in mortality starting around 30 days. Correlatively, 30 days of age is where results also show a sizeable increase in Smurf (SMF) proportions. Thus, activation of either Toll or IMD pathway leads to an increase in SMF proportions that are nicely paralleled to times of increased death in lifespan data. Drug-induction may be increasing mortality by hastening loss of barrier dysfunction. Although this mechanism is not yet well understood, many epithelial and protective matrixes are under strict regulation from the immune pathways (Buchon et al., 2009b). Additionally, chronic

increases in the Toll and IMD pathways may lead to desensitization of important regulatory pathways inside fly gut epithelia.

Intestinal physiology was assayed at 30 days of age, a time when flies undergoing immune induction were either on the precipice or in the midst of mass decline. Induction of either the Toll or IMD pathway resulted in acid-base disturbances at the level of the intestine in non-Smurf flies. From a naturally acidic compartment, the lumen of the intestine became more alkaline in nature. Changes in intestinal pH may be due to shifts in the microbiota populations and interactions between those differing microbial populations. Such shifts may thus result in varying bacterial metabolite release, triggering differential transcription and translation of cellular components. In other words, as microbe populations change with age so too will host-microbe interactions change. Increases in pH may perturb normal digestive processes, nutrient uptake, as well as water reabsorption within the intestine of the fly. Thus, leading to a decrease in number of excreta (Wayland et al., 2015). Further, mean lightness of fecal deposits was inversely correlated with mean area. As fecal concentration decreased mean area increased and vice versa. Interestingly, Toll and IMD activation had opposite results for mean lightness and mean area of fecal deposits. Previous studies have shown that loss of acid-secreting copper cells in the fly intestine leads to decompartmentalization of the microbes within the gut lumen (Keebaugh et al., 2016, Li et al., 2016). In other words, pH regulation of the intestine may be highly important in maintaining concentrations of microbes in specific pockets of the fly intestine. Changes in microbial density, specifically equilibration along the lumen, may hinder other

metabolic and digestive mechanisms from taking place. Thus, perpetuating intestinal and organismal decline.

Currently, we understand that increases in bacterial load and changes in bacterial composition are paralleled to, and to some extent cause, significant increases in immune activity (Rera et al., 2012, Clark et al., 2015). By manipulating the fly such that we force an increase in immune activity, we are able to assay the potential effects that age-related immune activation may have on bacteria in the gut. After only 10 days of immune activation, 20-day-old flies experience a substantial and significant increase in bacterial load. With the exception of a small, but significant, increase in Toll bacterial load data, there were no significant differences in bacterial load or composition between day 10 and day 20 uninduced control flies. The difference in age between day 10 and day 20 controls is not substantial enough to cause a huge increase in bacteria. By contrast, 10 days of immune induction is substantial enough to cause a 150 and 400 fold increase in bacterial load in drug induced 20-day-old flies. Although there were increases in overall bacterial load at 20 and 30 days of age, bacterial load, after induced immune activation, peaked at day 20 and decreased at day 30. Alphaproteobacteria levels follow the overall trend of bacterial load in Toll and IMD induced flies. However, at 30 days of age Toll induction causes a decrease in Alphaproteobacteria. Although not significant, there is a definite downward trend that may become significant upon experimental repeat. The IMD pathway controls release of an antimicrobial peptide responsible for containing gram-negative bacteria in the intestinal lumen (Buchon et al., 2009b). It may then be expected that IMD induction would cause substantial decreases in Gamma and Alphaproteobacteria, as these classes are largely gram-negative.

Although Gammaproteobacteria levels remain low, Alphaproteobacteria levels increase significantly at 20 and 30 days of age.

Interestingly, increases in Alphaproteobacteria are also seen immediately following loss of barrier function, or in Smurf flies (Clark et al., 2015). Changes in bacterial proportions in Smurfs and non-Smurfs may result in massive shifts between interacting microbes. However small, changes in bacterial cross talk may result in pathogenic responses. This may then further facilitate changes in and along gut epithelial cells, such as changes in tight junction expression and localization as well as AMP expression and interaction, ultimately leading to the demise of the organism. Other theories attribute age-related increases in immune activity to tolerant bacteria, which become desensitized to AMPs, and ultimately become harmful to the host (Jasper, 2015, Broderick, 2016). These theories only provide a subset of potential reasons for why changes in bacterial load and composition may lead to intestinal pathologies associated with the aging fly.

Changes in host-microbe dynamics are sufficient enough to deregulate organismal homeostasis. However, lifespan extensions in the fly have been seen upon control of hyperplasia, or abnormal cell division, and decreased proliferation of microbes in the gut (Clark et al., 2015, Guo et al., 2014). Additionally, preliminary immune knockdown experiments have shown potential life-extending properties. Thus, maintenance and conservation of the bacterial lumen and epithelia is critically important for organismal health and survival. Potential hypotheses for why flies experience an increase in lifespan during these manipulations include: microbiota interactions in the gut, AMP interactions in and along fly epithelial cells, and interactions between

microbiota and AMPs. Keeping bacterial levels low, by preventing AMP induction, may help antibacterial molecules better target and trigger lysis of particular bacteria. Mechanistically, AMPs utilize electrostatic interactions to perturb cell membrane cohesion causing bacterial lysis (Zhang et al. 2016). These results also help elucidate the potential harm caused directly by AMP over expression on intestinal epithelia. Future work will be aimed at better understanding the effects of microbial imbalance in the intestine as well as the individual and synergistic roles that the immune system and bacteria play in regulating or deregulating organism health.

Figure 1

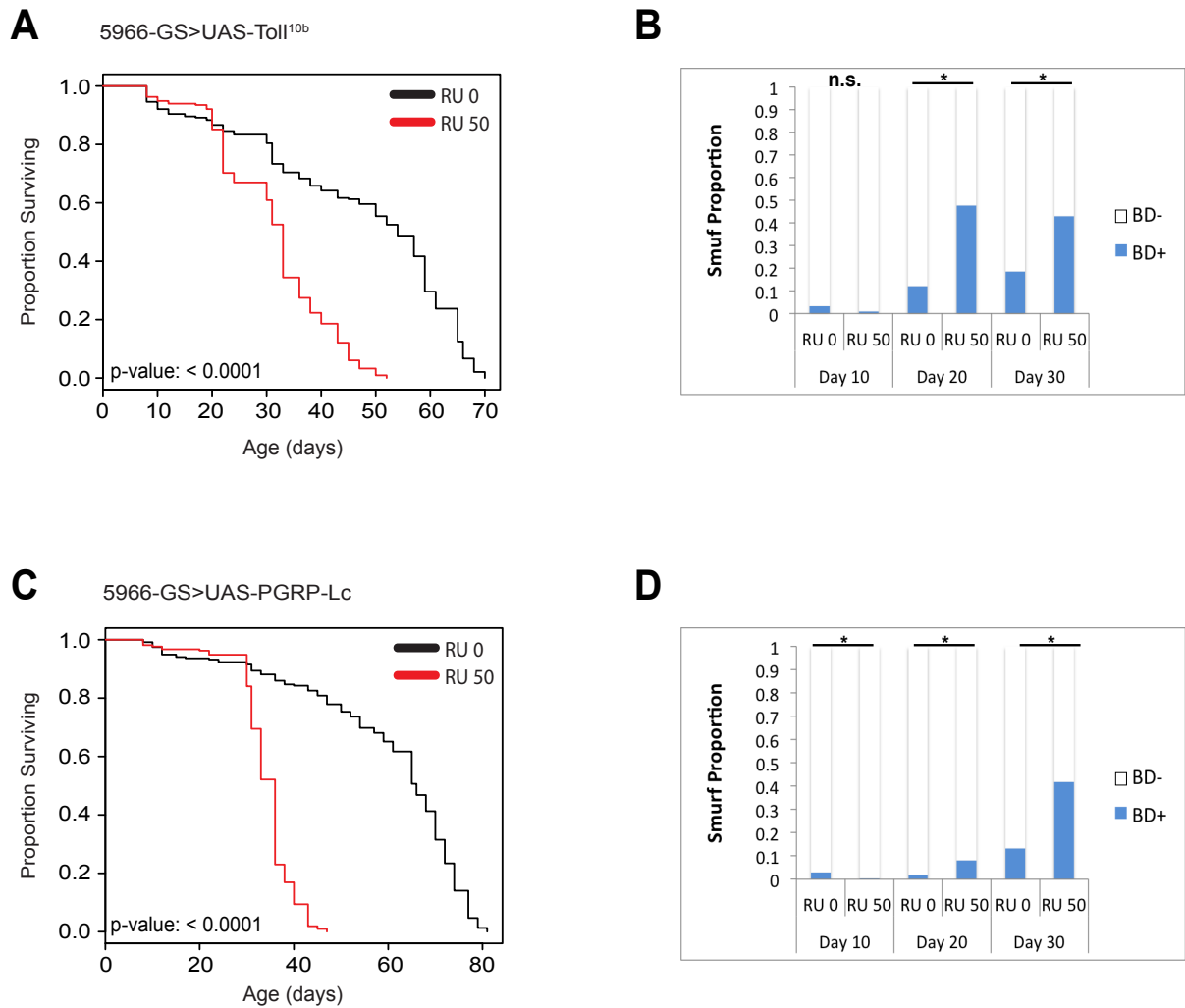


Figure 1. Activation of Immune Pathways Leads to Increased Barrier Dysfunction and Increased Mortality

Lifespan curves (A and C) and Smurf proportions (B and D) in UAS-Toll^{10b}/5966-Geneswitch (A and B) and UAS-PGPR-LC/5966-Geneswitch (C and D) female flies drug induced from day 10 of adulthood (RU 50) and uninduced controls (RU 0). n = >200 flies/condition. BD-, non-Smurf; BD+, Smurf. Log rank test for survival data was used in (A) and (C), binomial test for Smurf proportions was used in (B) and (D). Smurf proportion p-values: (B) Day 10 = 0.055, Day 20 = <2.2e-16, Day 30 = 2.93e-12; (D) Day 10 = 0.012, Day 20 = 5.13e-8, Day 30 = <2.20e-16. p-value <0.05 = *

Figure 2

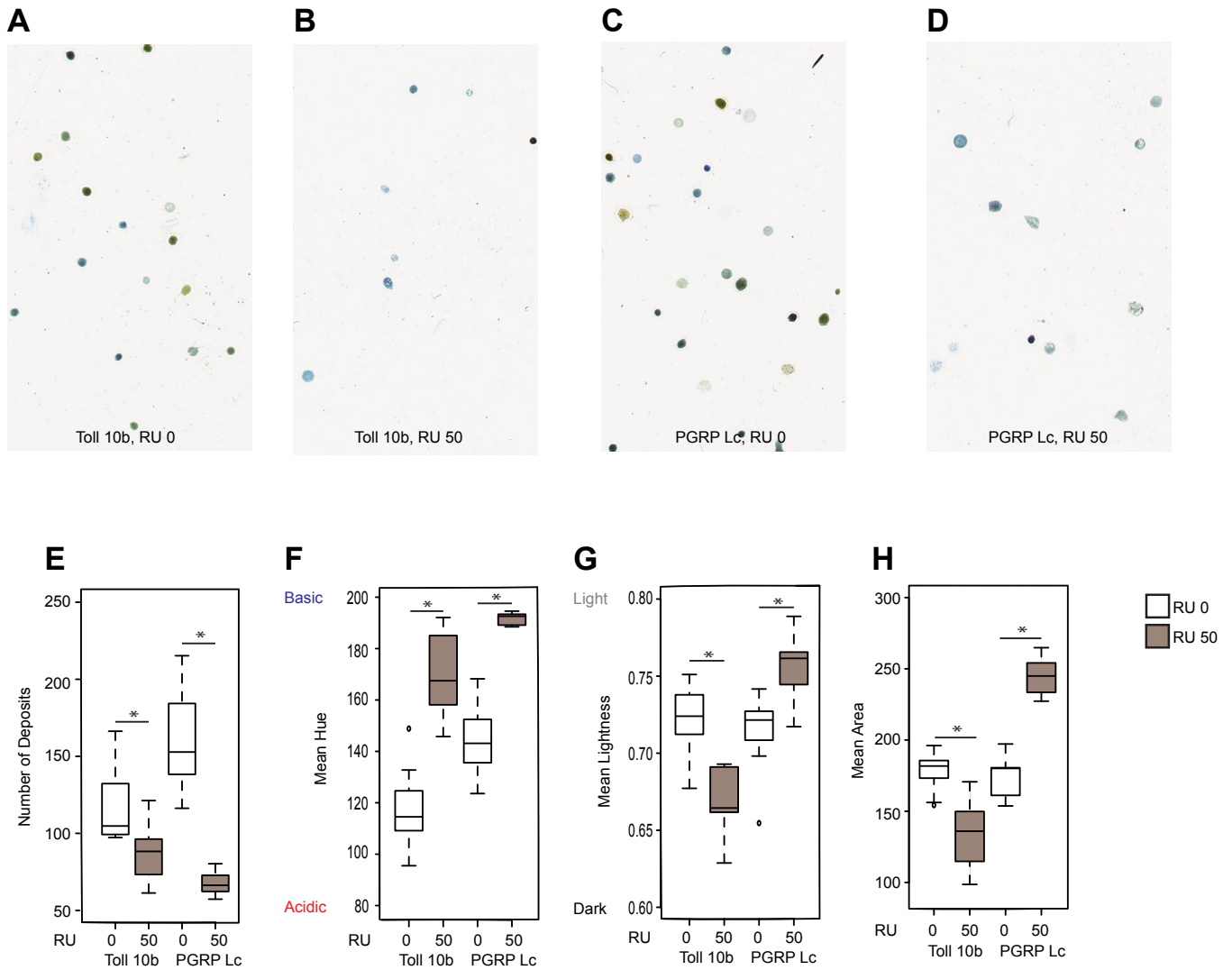


Figure 2. Immune Activation Impairs Digestive Transit

Representative images (A-D) and analysis (E-H) of fecal output over a 24-hr period from drug induced (RU 50) and uninduced controls (RU 0) female non-Smurf flies at 30 days of age. Shown for both UAS-Toll^{10b}/5966-Geneswitch and UAS-PGRP-LC/5966-Geneswitch are number (E), mean hue (F), mean lightness (G), and mean area (H) of deposits. n = 10 replicate groups of 10 flies. Boxplots display the first and third quartile, with the horizontal bar at the median. Mann-Whitney U Test was used in (E-H). Fecal analysis p-values for Toll^{10b} and PGRP Lc respectively: (E) 0.002, 0.0001; (F) 0.0001, 0.0001; (G) 0.0001, 0.006; (H) 0.0001, 0.0001. p-value <0.05 = *. Toll^{10b} = 5966-GS>UAS-Toll^{10b}, PGRP-LC = 5966-GS>UAS-PGRP-LC

Figure 3

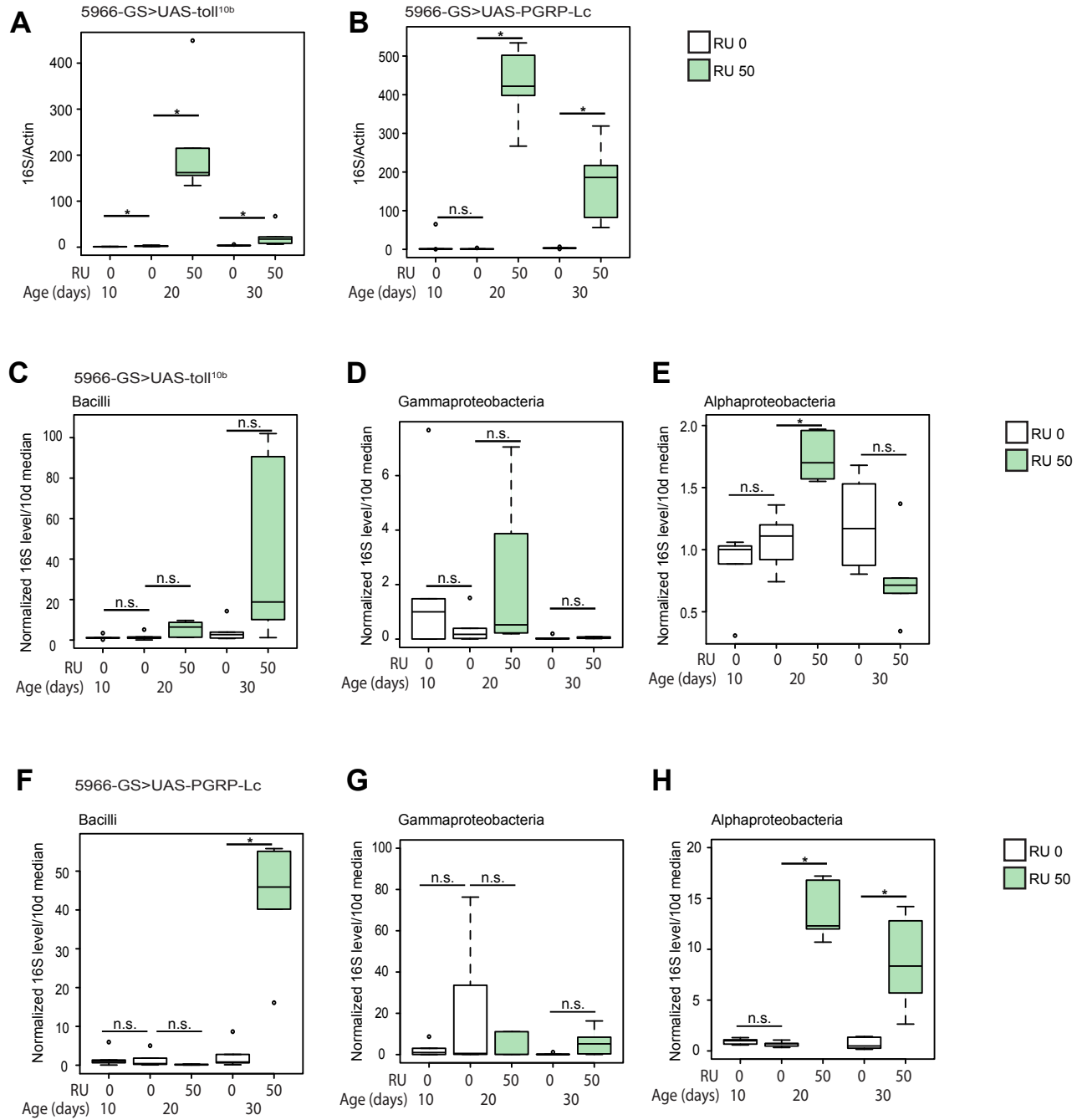


Figure 3. Immune Activation Changes Whole Fly Bacterial Load and Bacterial Composition

(A and B) Bacterial levels assayed by qPCR of 16S with universal primers in drug induced (RU 50) and uninduced controls (RU 0) non-Smurfs at 10, 20, and 30 days of age. UAS-Toll^{10b}/5966-Geneswitch (A) and UAS-PGPR-LC/5966-Geneswitch (B). (C-H) Bacterial levels assayed by taxon-specific qPCR of the 16S rRNA gene in non-Smurfs at 10, 20, and 30 days of age. n = 5 replicates of 5 surface-sterilized whole flies. UAS-Toll^{10b}/5966-Geneswitch (C-E) and UAS-PGPR-LC/5966-Geneswitch (F-H). Boxplots display the first and third quartile, with the horizontal bar at the median. Wilcoxon Test was used in (E-H). Bacterial level p-values for Day 10 vs. Day 20 RU 0 | Day 20 RU 0 vs. RU 50 | Day 30 RU 0 vs. RU 50: (A) 0.014, 0.0079, 0.0079; (B) 0.5368, 0.0079, 0.0119; (C) 1.000, 0.0952, 0.0952; (D) 0.7922, 0.1508, 0.4206; (E) 0.329, 0.0079, 0.0556; (F) 0.7922, 0.1508, 0.0079; (G) 0.5368, 1.000, 0.0952; (H) 0.2468, 0.0079, 0.0079.

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