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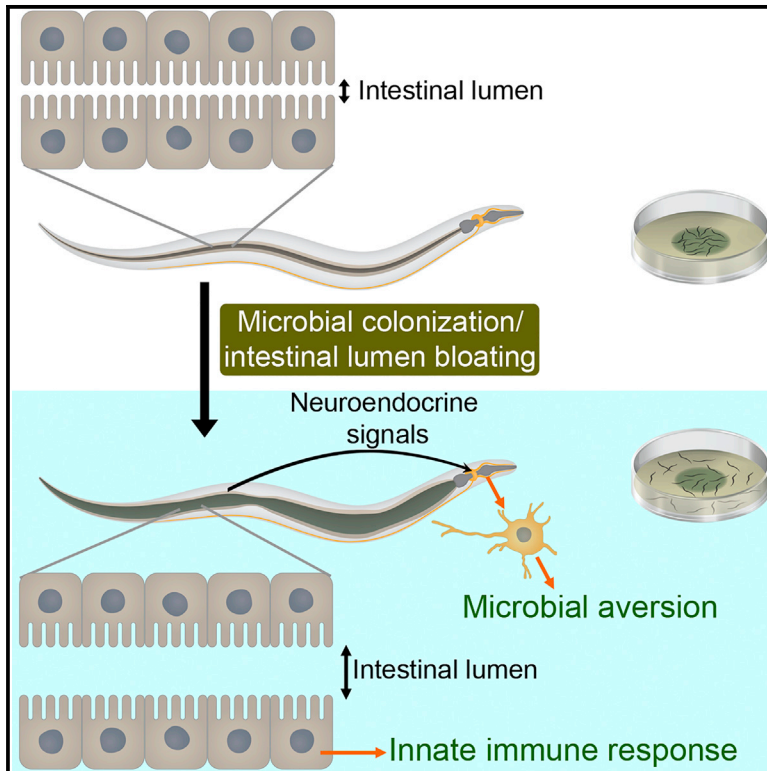
Microbial Colonization Activates an Immune Fight-and-Flight Response via Neuroendocrine Signaling

Graphical Abstract

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In Brief
Singh and Aballay describe a mechanism by which a fight-and-flight response against pathogenic microbes is activated. They show that in *C. elegans* microbial colonization induces bloating of the intestinal lumen, which enhances the expression of innate immune genes and neuroendocrine pathway genes required for the elicitation of a microbial aversion behavior.

Highlights

- *P. aeruginosa* colonization elicits immune genes and pathogen aversion in *C. elegans*

- Bloating of the intestinal lumen by microbial colonization upregulates immune genes

- Intestinal bloating upregulates neuroendocrine pathways, inducing a flight response

- NPR-1 signaling is required for a flight response that elicits pathogen aversion

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Microbial Colonization Activates an Immune Fight-and-Flight Response via Neuroendocrine Signaling

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SUMMARY

The ability to distinguish harmful and beneficial microbes is critical for the survival of an organism. Here, we show that bloating of the intestinal lumen of *Caenorhabditis elegans* caused by microbial colonization elicits a microbial aversion behavior. Bloating of the intestinal lumen also activates a broad innate immune response, even in the absence of bacterial pathogens or live bacteria. Neuroendocrine pathway genes are upregulated by intestinal bloating and are required for microbial aversion behavior. We propose that microbial colonization and bloating of the intestine may be perceived as a danger signal that activates an immune fight-and-flight response. These results reveal how inputs from the intestine can aid in the recognition of a broad range of microbes and modulate host behavior via neuroendocrine signaling.

INTRODUCTION

Organisms live in complex environments replete with potential dangers. The ability to distinguish potential dangers from useful resources is critical for the survival of an organism. Discrimination of microbial pathogens from commensal organisms by the host is one such example (Blander and Sander, 2012). To effectively counteract microbial pathogens, metazoans have evolved mechanisms to recognize them and deploy counter attacks at molecular and behavioral levels. Potential pathogenic microorganisms are detected by receptors that recognize microbial-associated molecular patterns (MAMPs) (Stuart et al., 2013). In addition, the recognition of endogenous signals, or damage-associated molecular patterns (DAMPs), that result from the cellular damage caused by infections seems to play an essential role in the activation of innate immune responses (Matzinger, 2002). A reliance on specific cellular receptors that are capable of sensing specific microbial patterns limits the range of pathogens that a given host may be able to detect. In contrast, host physiological mechanisms that are capable of sensing alterations caused by infections provide a broad response to microbial pathogens.

*Caenorhabditis elegans* is a free-living nematode that forages on decomposing organic matter rich in bacteria (Schulenburg and Félix, 2017). Behaviors of *C. elegans* as diverse as feeding, locomotion, and aerotaxis are affected by the exposure to different bacterial species (Bretscher et al., 2011; Sawin et al., 2000; Schulenburg and Félix, 2017). In addition, several pathogens that are present in the natural habitat of *C. elegans* can impair growth and induce stress (Samuel et al., 2016). Pathogenic bacteria infect and kill *C. elegans* and elicit a protective avoidance behavior, which is a crucial defense response employed by the animal (Martin et al., 2017; Meisel and Kim, 2014; Pujol et al., 2001; Styer et al., 2008). Bacterial metabolites, such as serrawettin W2 from *Serratia marcescens*, have been implicated in elicitation of the pathogen avoidance behavior (Pradel et al., 2007). Similarly, the secondary metabolites phenazine-1-carboxamide and pyochelin from *Pseudomonas aeruginosa* have been shown to activate neuroendocrine signaling, potentially leading to elicitation of the avoidance behavior (Meisel et al., 2014).

In this study, we determined that different bacterial pathogens elicit different patterns of avoidance in *C. elegans*. We found that while certain pathogens elicited a rapid avoidance behavior that could be observed within 5 minutes of exposure, other pathogens induced a late avoidance behavior that was only evident after 12 hours of exposure. This late avoidance behavior was induced by bacterial colonization and bloating of the intestinal lumen of *C. elegans*, which also led to the activation of the expression of innate immune genes. The increased aversion required a neuroendocrine signaling that was activated by bloating of the intestinal lumen. We propose that the bloating-induced fight-and-flight (BIFF) response (referred to as BIFF response) is a general mechanism used by *C. elegans* to activate immune pathways and elicit microbial aversion.

RESULTS

P. aeruginosa Colonization of the Intestine Induces Pathogen Avoidance

Because behavioral pathogen avoidance is a crucial defense response employed by *C. elegans* and significantly improves the survival of animals (Martin et al., 2017; Meisel and Kim, 2014; Reddy et al., 2009; Styer et al., 2008), we focused on elucidating the mechanism by which this behavior is elicited. We exposed the animals to the lawns of different bacterial pathogens, including *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *P. aeruginosa*, and *S. marcescens*. While the animals were initially attracted towards the lawns of the Gram-negative
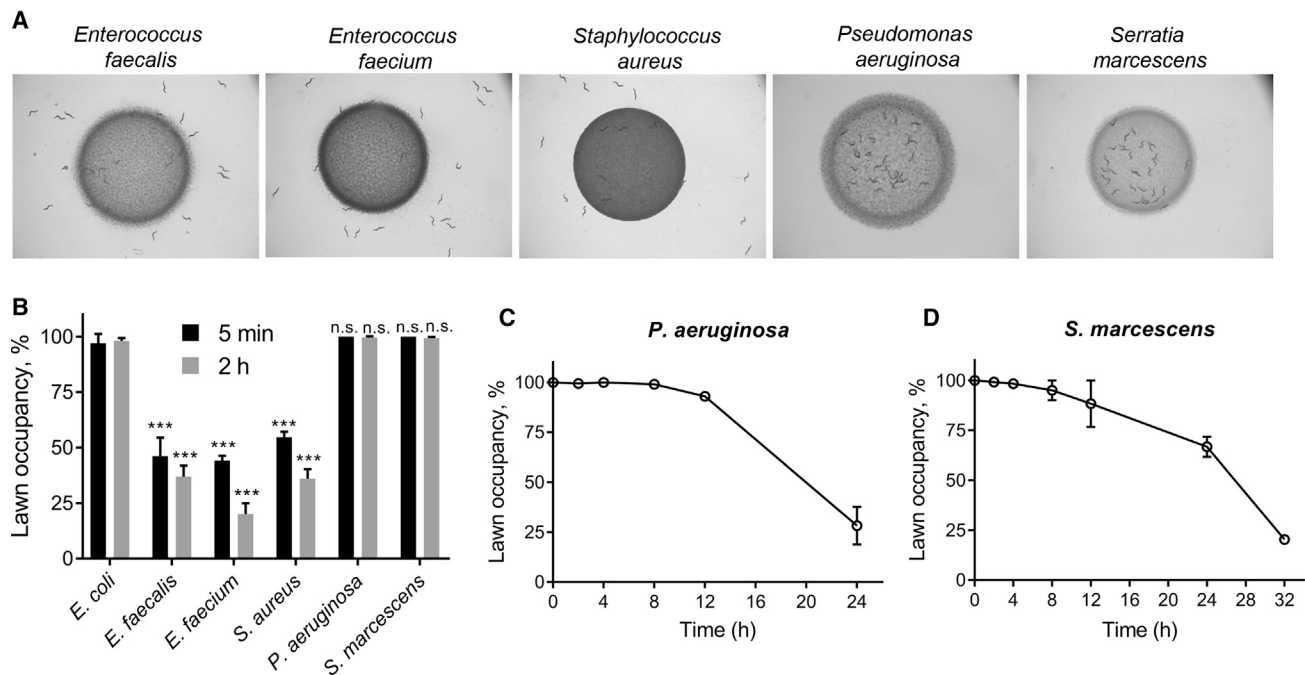
Figure 1. Microbial Pathogens Elicit Different Patterns of Avoidance in *C. elegans*
(A) Distribution of wild-type N2 *C. elegans* on lawns of different pathogenic bacteria. Representative images are shown at 2 h after transfer of *C. elegans* to the bacterial lawns.
(B) Occupancy of different bacterial lawns by N2 animals at 5 min and 2 h after transfer to the lawns. p values for *E. faecalis*, *E. faecium*, *S. aureus*, *P. aeruginosa*, and *S. marcescens* are relative to *E. coli* for the corresponding time points. ***$p < 0.001$ via the t test. n.s., non-significant.
(C and D) Occupancy of *P. aeruginosa* (C) and *Serratia marcescens* (D) lawns by *C. elegans* over time.
See also Figure S1.

pathogens *P. aeruginosa* and *S. marcescens*, they avoided the lawns of the Gram-positive pathogens *E. faecalis*, *E. faecium*, and *S. aureus* (Figures 1A and 1B). Colonization of the intestine by these pathogens is only observed after several hours of exposure (Garsin et al., 2001; Irazoqui et al., 2010; Yuen and Ausubel, 2018). Thus, the rapid avoidance phenotype that is observed after 5 minutes of pathogen exposure seems to be independent of microbial colonization of the intestine. The animals continued to avoid the lawns of *E. faecalis*, *E. faecium*, and *S. aureus* after longer durations of incubation (Figure S1A), indicating that *C. elegans* actively avoided these bacteria throughout the course of the infection. In contrast to its rapid avoidance of the studied Gram-positive pathogens, *C. elegans* only began to avoid the lawns of *P. aeruginosa* and *S. marcescens* after 12 hours of incubation (Figures 1C and 1D), as shown in previous studies (Pujol et al., 2001; Sun et al., 2011). These results suggest that while pathogen avoidance directed towards *E. faecalis*, *E. faecium*, and *S. aureus* is rapidity elicited, avoidance of *P. aeruginosa* and *S. marcescens* develops after a period of interaction between the host and these pathogens.

To gain insight into the host mechanism(s) involved in the elicitation of the late avoidance behavior exhibited by *C. elegans*, we focused on the effects of *P. aeruginosa* on the animals. We studied the rate of colonization of the *C. elegans* intestine by *P. aeruginosa*. As shown in Figure 2A, the kinetics of colonization of the *C. elegans* intestine by *P. aeruginosa* was similar to the kinetics of avoidance of the pathogen. To study the relationship

between colonization and avoidance behavior, we analyzed the bacterial colonization of animals present inside and outside of the lawn after 18 hours of incubation on *P. aeruginosa* expressing green fluorescent protein (GFP). We found that the animals that had left the lawn showed higher levels of colonization than those that remained on the lawn at the same time point (Figures 2B and 2C), suggesting that colonization of the intestine may induce the avoidance behavior. We reasoned that if the colonization of the intestine indeed induces the avoidance behavior, then animals that resist colonization should exhibit diminished avoidance behavior. To test this hypothesis, we studied the avoidance behavior of animals after knockdown of the gene *nol-6*. Previous studies have shown that RNA interference (RNAi)-mediated knockdown of *nol-6*, a nucleolar RNA-associated protein, prevents colonization of the intestine by microbial pathogens, including *P. aeruginosa* (Fuhrman et al., 2009). Consistent with the idea that colonization results in the elicitation of avoidance behavior, inhibition of *nol-6* blocked the pathogen-avoidance phenotype of *P. aeruginosa* (Figures 2D and 2E) but not the rapid avoidance of Gram-positive pathogens (Figure S1B). Taken together, these results suggest that the intestinal colonization by *P. aeruginosa* elicits the observed avoidance behavior.

Bloating of the Intestinal Lumen Enhances the Microbial Aversion Behavior

Next, we tested whether increased colonization of the intestine could enhance the avoidance behavior. We studied animals
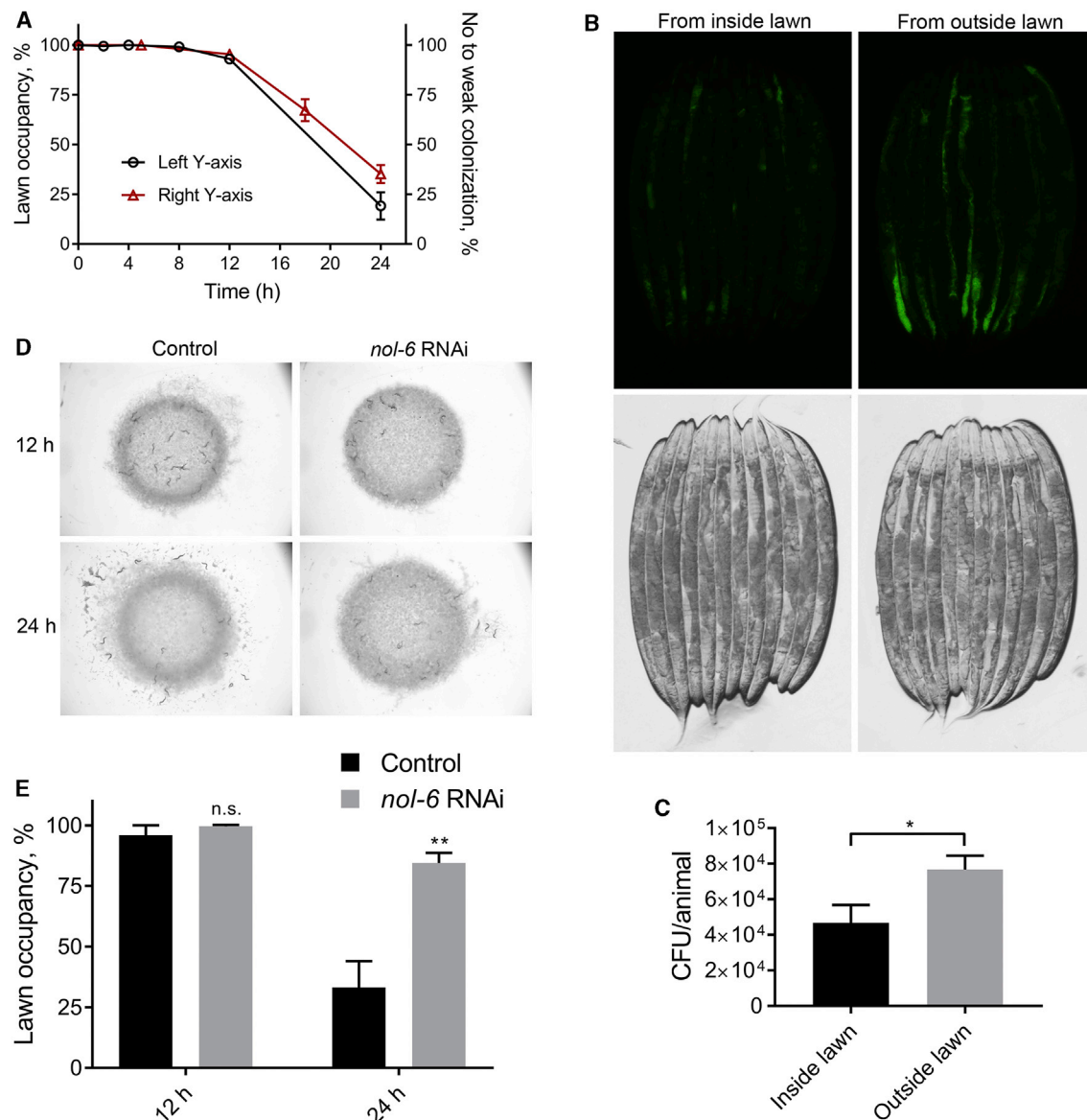
Figure 2. *P. aeruginosa* Colonization of the Intestine Induces Pathogen Avoidance

(A) Percent occupancy of N2 animals on *P. aeruginosa* lawn over time (left y axis) and percent of N2 animals with no or weak colonization of *P. aeruginosa*-GFP over time (right y axis). Animals showing colonization in a small fraction of the intestine were considered weakly colonized.

(B) Representative photomicrographs of animals that were picked either from the inside or outside of *P. aeruginosa*-GFP lawn after 18 h of incubation on the lawns.

(C) Colony-forming units per animal of animals that were picked either from the inside or outside of *P. aeruginosa*-GFP lawn after 18 h of incubation on the lawns. $*p < 0.05$ via the t test.

(D) Representative photomicrographs of the distribution of control as well as *nol-6* RNAi animals after 12 h and 24 h of incubation on *P. aeruginosa* lawns.

(E) Percent occupancy of the control as well as *nol-6* RNAi animals after 12 h and 24 h of incubation on *P. aeruginosa* lawns. $**p < 0.01$ via the t test. n.s., non-significant.

See also Figure S1.

that are defective in the defecation motor program (DMP), and are prone to bacterial colonization (Shapira and Tan, 2008). The genes *aex-5*, *flr-1*, *nhx-2*, and *pbo-1* were selected for the current study because their inhibition is known to lead to defects in the DMP, which results in the bloating of the intestinal lumen by the accumulation of bacteria (Branicky and Hekimi, 2006; Pfeiffer et al., 2008; Thomas, 1990). As expected, defects in

the DMP led to enhanced accumulation of *P. aeruginosa* (Figures 3A and S2A). Animals with bloated lumens showed enhanced avoidance of *P. aeruginosa* lawns, and within 2 h of the transfer, the animals were observed to avoid the *P. aeruginosa* lawns (Figure 3B). Additional mutants that lead to mild defects in the DMP also showed enhanced *P. aeruginosa* accumulation (Figures S2B and S2C) and exhibited enhanced avoidance behavior
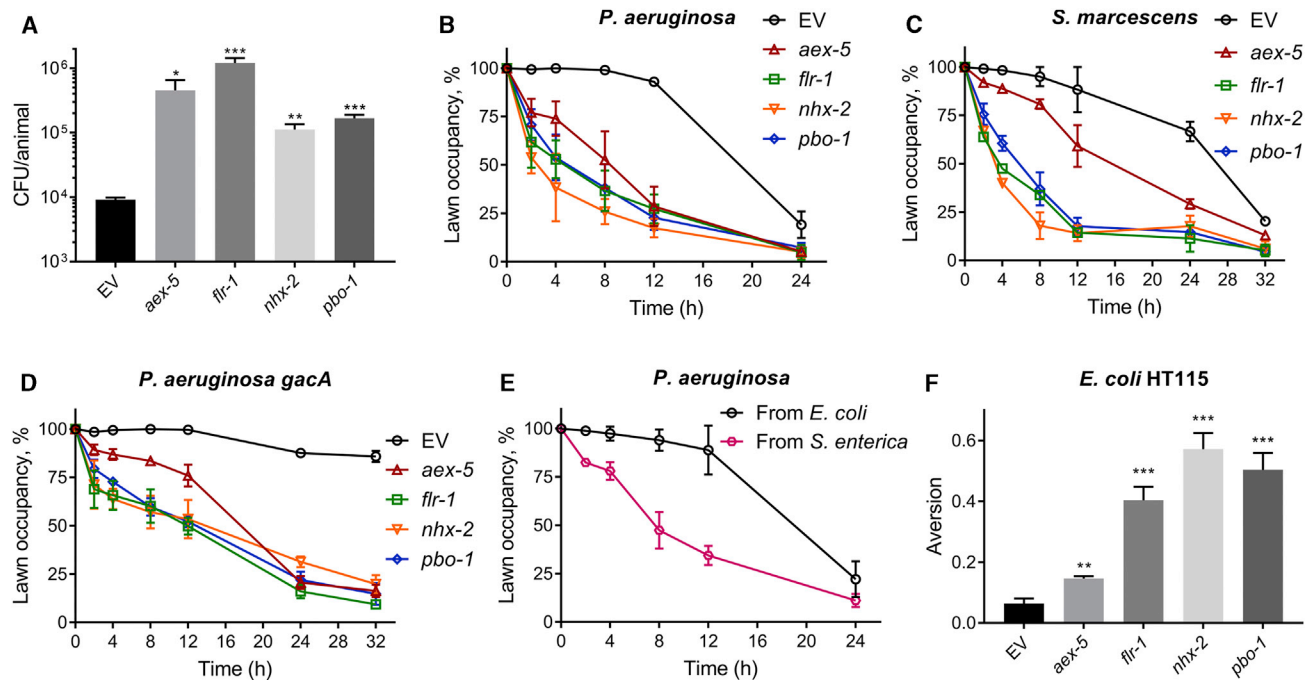
Figure 3. Microbial Colonization of the Intestine Leads to Enhanced Microbial Aversion Behavior

(A) Colony-forming units per animal for N2 animals grown on control as well as *aex-5*, *flr-1*, *nhx-2*, and *pbo-1* RNAi followed by exposure to *P. aeruginosa*-GFP for 5 h at 25°C. EV, empty vector RNAi control. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ via the t test.

(B–D) Percent occupancy of *P. aeruginosa* (B), *Serratia marcescens* (C), and *P. aeruginosa gacA* mutant (D) lawns over time by N2 animals grown on control, as well as *aex-5*, *flr-1*, *nhx-2*, and *pbo-1* RNAi. EV, empty vector RNAi control.

(E) Percent occupancy of *P. aeruginosa* lawns over time by N2 animals grown on *S. enterica* for 48 h post L4 larval stage. The control animals were grown on *E. coli*.

(F) Aversion to *E. coli* HT115 upon knockdown of *aex-5*, *flr-1*, *nhx-2*, and *pbo-1* by RNAi. ***$p < 0.001$ and **$p < 0.01$ via the t test.

See also Figures S2 and S3.

(Figure S2D). Animals with bloated lumens also showed enhanced avoidance of the pathogen *S. marcescens* (Figure 3C).

Animals that are defective in feeding manifest pharyngeal anomalies (Avery, 1993; Straud et al., 2013), which lead to enhanced bacterial colonization (Portal-celhay et al., 2012). Thus, we studied several feeding- defective mutants to explore the relationship between bacterial colonization and avoidance behavior further. As shown in Figures S2E and S2F, six out of eight tested mutants exhibited enhanced accumulation of *P. aeruginosa*. Consistent with the idea that bacterial colonization elicits an avoidance behavior, all of the animals that had enhanced bacterial accumulation also showed enhanced avoidance of *P. aeruginosa* (Figure S2G). On the other hand, *eat-5 (ad1402)* and *eat-7(ad450)* animals did not exhibit enhanced *P. aeruginosa* accumulation or enhanced avoidance behavior (Figures S2E–S2G). Taken together, these results suggested that animals with bloated lumens show enhanced avoidance of the pathogen *P. aeruginosa*.

Non-virulent strains of *P. aeruginosa* with mutations in the virulence factor *gacA* do not cause infection and fail to colonize the intestine of *C. elegans* (Tan et al., 1999). As shown previously (Hao et al., 2018), the *gacA* mutant of *P. aeruginosa* does not elicit an avoidance behavior in *C. elegans* (Figure S3). *P. aeruginosa gacA* mutants are also deficient in the production of the secondary metabolites phenazines and pyochelin, which

have been suggested to be important for elicitation of the avoidance behavior in *C. elegans* (Meisel et al., 2014). Thus, the inability of *P. aeruginosa gacA* mutants to elicit an avoidance behavior in *C. elegans* could be because of either their inability to colonize the intestine or a lack of production of secondary metabolites or virulence factors that are important for eliciting the avoidance behavior. We found that animals with bloated lumens started to avoid the lawns of *P. aeruginosa gacA* within only two hours of transfer, and approximately 75% of the animals were outside the lawns by 24 hours of incubation (Figure 3D). In contrast, control animals showed no avoidance even at 32 hours after transfer to *P. aeruginosa gacA* lawns (Figure 3D). These results suggest that intestinal colonization and bloating of the lumen play an important role in elicitation of the pathogen avoidance behavior.

Next, we tested whether colonization and bloating of the *C. elegans* intestinal lumen by a given pathogen could elicit avoidance to another pathogen. We transferred larval L4 animals to the lawns of *Salmonella enterica* for 48 hours. We used *S. enterica* because infection by this pathogen leads to persistent colonization and bloating of the intestinal lumen, but it does not elicit an avoidance behavior (Fuhrman et al., 2009; Tenor and Aballay, 2008). Wild-type animals grown on *S. enterica* showed increased avoidance of *P. aeruginosa* compared with control animals grown on *E. coli* (Figure 3E). Finally, we tested the aversive behavior of
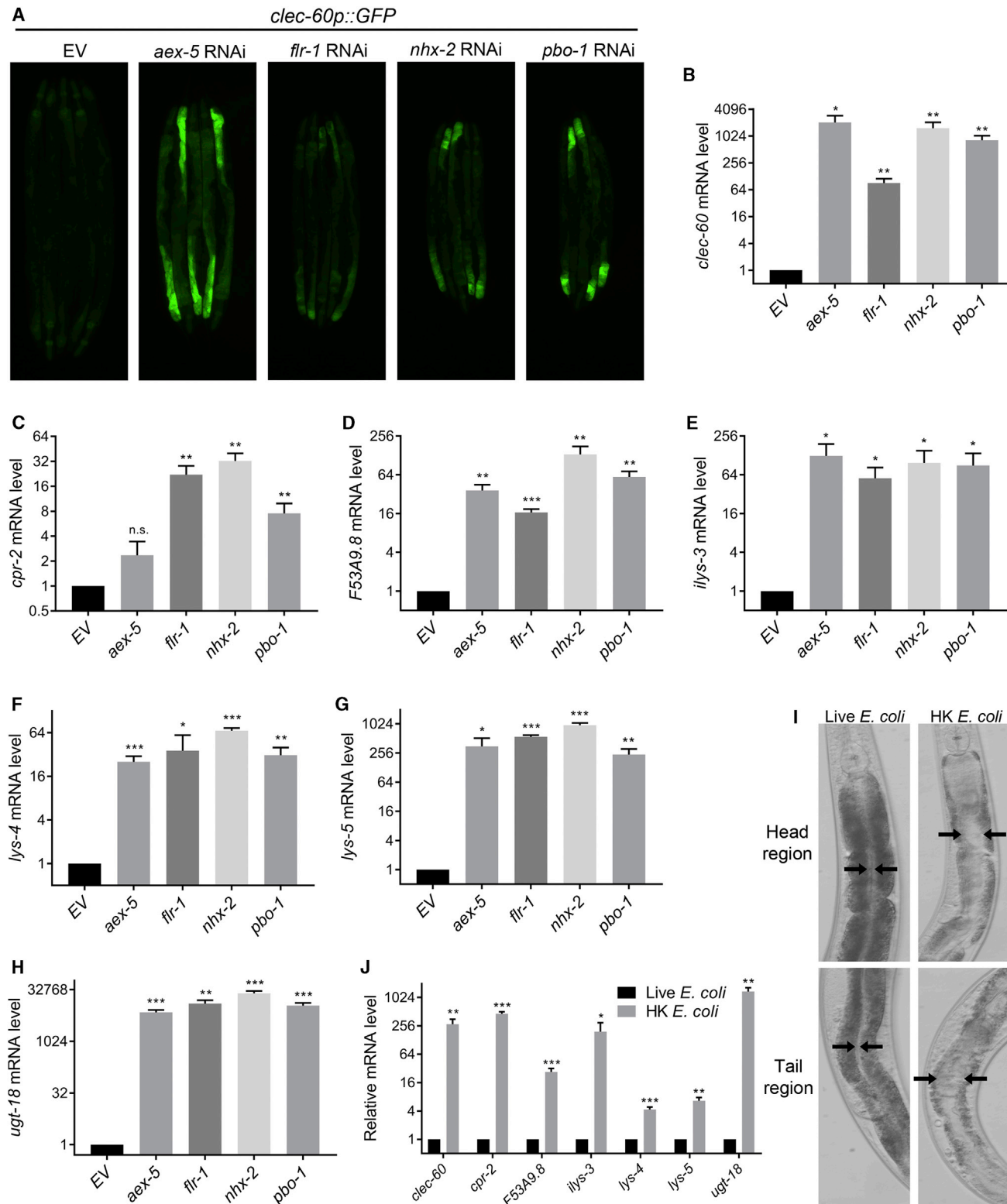
Figure 4. Intestinal Lumen Bloating Activates Innate Immune Genes

(A) Representative photomicrographs of *clec-60p::GFP* animals grown on RNAi control bacteria, as well as bacteria for RNAi against *aex-5*, *flr-1*, *nhx-2*, and *pbo-1*. EV, empty vector RNAi control.

(legend continued on next page)

animals with bloated intestinal lumens towards the non-pathogenic bacteria *E. coli*. Animals with bloated lumens showed an increased aversion to *E. coli* (Figure 3F). It is not known whether there is an attractant on the *S. enterica* lawn (Fuhrman et al., 2009; Tenor and Aballay, 2008) that may override the bloating-induced aversion. Currently, *S. enterica* appears to be the exception and not the rule.

Bloating of the Intestinal Lumen Activates a Broad Innate Immune Response

C. elegans is capable of mounting a distinct transcriptional response against different bacterial pathogens (Wong et al., 2007). However, diverse bacterial pathogens, including *E. faecalis*, *E. faecium*, *S. aureus*, *S. marcescens*, and *Photorhabdus luminescens*, also activate the expression of the same innate immune genes (Irazoqui et al., 2010; Wong et al., 2007; Yuen and Ausubel, 2018). The aforementioned bacterial pathogens all cause colonization and bloating of the *C. elegans* intestinal lumen. Thus, we reasoned that animals might sense bloating of the intestinal lumen as a potential pathogenic attack and trigger a defense response not only by eliciting pathogen avoidance but also by activating innate immune genes. In such a case, bloating of the intestine by defects in the DMP would trigger the activation of innate immune genes in the absence of pathogens.

To address whether bloating of the *C. elegans* intestinal lumen on *E. coli* can engage transcriptional responses that are only observed upon exposure to pathogens, we examined the expression of the genes *clec-60*, *cpr-2*, *F53A9.8*, *ilys-3*, *lys-4*, *lys-5*, and *ugt-18*, which are induced by two or more of the bacterial pathogens capable of colonizing the intestine of the animals (Irazoqui et al., 2010; Wong et al., 2007; Yuen and Ausubel, 2018). A reporter strain expressing GFP under the promoter of *clec-60* was induced in the DMP-defective animals on *E. coli* (Figure 4A). The endogenous mRNA levels of *clec-60* were also induced in the DMP-defective animals (Figure 4B). Similarly, the immune genes *cpr-2*, *F53A9.8*, *ilys-3*, *lys-4*, *lys-5*, and *ugt-18* showed significantly increased levels in the DMP-defective animals compared with the control animals (Figures 4C–4H). Several of these immune genes have been shown to be important for the survival of *C. elegans* on pathogenic bacteria (Gravato-Nobre et al., 2016; Irazoqui et al., 2010; Luhachack et al., 2012). Therefore, the upregulation of these genes will likely improve the survival of *C. elegans* on pathogenic bacteria. However, investigations of the survival of animals with defects in the DMP on pathogens would not be informative because these animals are known to accumulate pathogens, resulting in an enhanced susceptibility to infection. We reasoned that the animals with bloated intestinal lumens, which exhibit an upregulated immune response, might show increased survival

on *E. coli* since the latter can be mildly pathogenic, especially as the animals age (Garigan et al., 2002; Podshivalova et al., 2017). Indeed, animals with defects in the DMP showed a significantly enhanced lifespan compared with control animals grown on *E. coli* (Figure S4).

Next, we sought to address whether bloating of the intestinal lumen in the absence of RNAi for DMP genes or bacterial infection upregulated the expression of immune genes. We found that animals that were fed heat-killed *E. coli* (HK *E. coli*) exhibited bloated intestinal lumens (Figure 4I). The pharyngeal pumping rate and the length of the DMP cycle of animals that were fed HK *E. coli* was comparable to that of animals fed live bacteria (Figures S5A and S5B), suggesting that the viscous lawn of HK *E. coli* may mechanically block the intestine. As shown in Figure 4J, animals fed on HK *E. coli* had significantly higher levels of mRNAs for innate immune genes compared with control animals grown on live *E. coli*. Taken together, these results suggest that bloating of the intestinal lumen may be perceived as a danger signal that induces a fight-and-flight response.
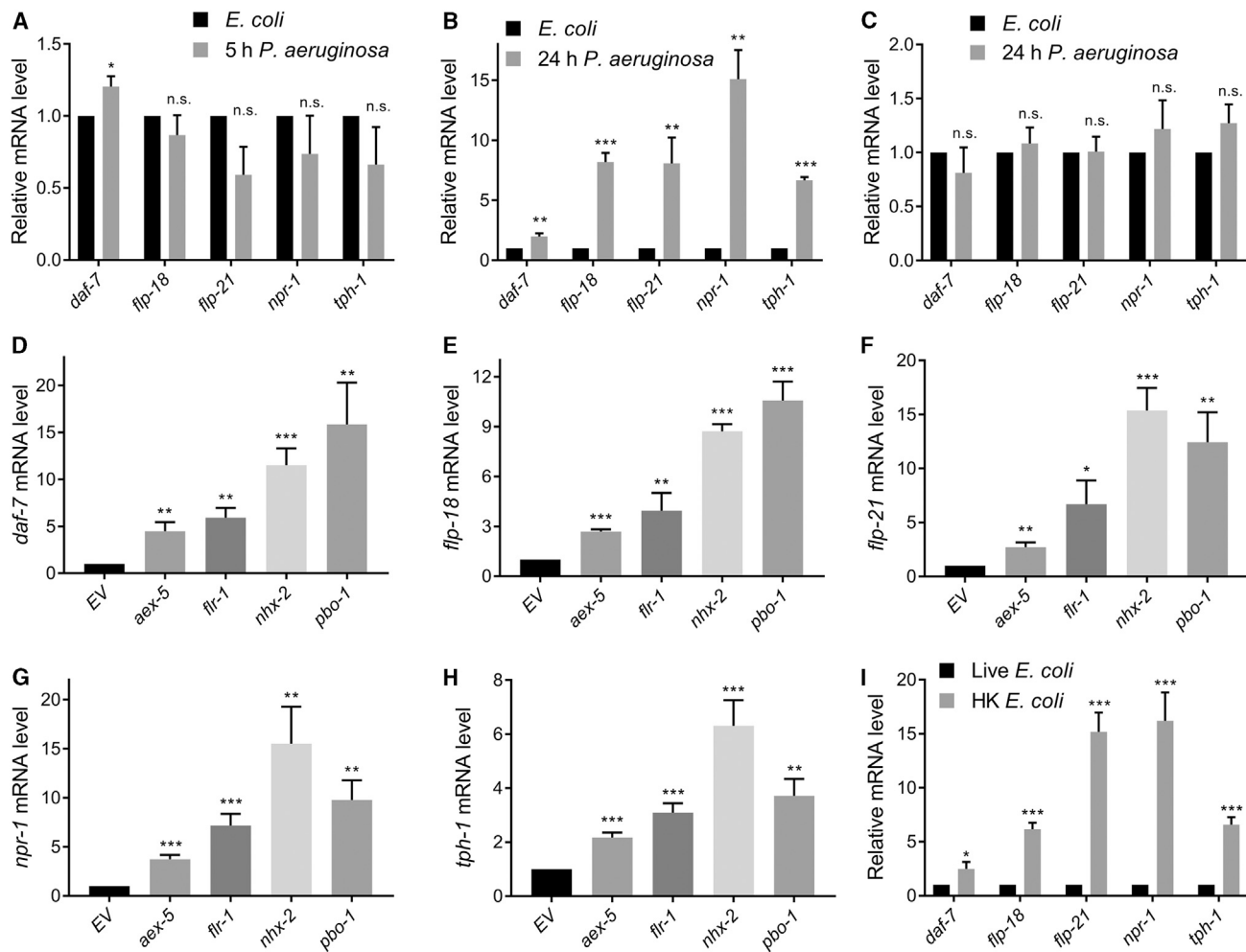
Intestinal Lumen Bloating Enhances the Expression of Neuroendocrine Signaling Pathway Genes

Neurosensory inputs are necessary for pathogen avoidance behavior (Cao et al., 2017; Chang et al., 2011; Pradel et al., 2007; Reddy et al., 2009; Styer et al., 2008); however, how animals integrate information to modulate neuroendocrine signaling pathways remains largely unknown. To test whether colonization and bloating of the intestinal lumen may modulate neuroendocrine signaling, we studied the gene expression of different components of neuroendocrine pathways that might influence the avoidance behavior, including the NPR-1/GPCR pathway (*npr-1*, *flp-18*, and *flp-21*) (Styer et al., 2008), the DAF-7/TGF-β pathway (Meisel et al., 2014), and the serotonin biosynthesis pathway (Shivers et al., 2009). We found that the expression of genes of the aforementioned pathways did not change after 5 h of exposure to *P. aeruginosa* (Figure 5A). However, all the studied genes were upregulated after 24 h of *P. aeruginosa* exposure (Figure 5B). To test whether the increased expression observed after 24 h of *P. aeruginosa* infection was related to the colonization of the intestine rather than a longer exposure alone, we took advantage of *nol-6* RNAi animals. These animals, which resist intestinal colonization up to 24 h after *P. aeruginosa* infection (Fuhrman et al., 2009), did not develop an aversion to the pathogen (Figures 2C and 2D). Figure 5C shows no changes in gene expression after 24 h of *P. aeruginosa* infection in *nol-6* RNAi animals in comparison to control animals grown on *E. coli*. These results suggested that colonization of the intestine by *P. aeruginosa* is required to induce neuroendocrine pathways linked to the pathogen avoidance behavior.

(B–H) Quantitative reverse transcription-PCR (qRT-PCR) for the immune genes *clec-60* (B), *cpr-2* (C), *F53A9.8* (D), *ilys-3* (E), *lys-4* (F), *lys-5* (G), and *ugt-18* (H) in N2 animals grown on control, as well as *aex-5*, *flr-1*, *nhx-2*, and *pbo-1* RNAi. EV, empty vector RNAi control. ***p < 0.001, **p < 0.01, and *p < 0.05 via the t test. n.s., non-significant.

(I) Representative photomicrographs of N2 animals grown on live *E. coli* until the young adult stage, followed by incubation on HK *E. coli* for 24 h at 20°C. The control animals were maintained on live *E. coli*. Representative photomicrographs of the head and tail regions are shown. Arrows point to the border of the intestinal lumen.

(J) Gene expression levels for the immune genes in N2 animals grown on live as well as HK *E. coli*. The animals were grown on live *E. coli* until the young adult stage before incubation on HK *E. coli* for 24 h at 20°C. ***p < 0.001, **p < 0.01, and *p < 0.05 via the t test.

See also Figures S4 and S5.

Figure 5. Intestinal Lumen Bloating Enhances the Expression of Neuroendocrine Signaling Pathways

(A and B) Gene expression analysis of N2 animals grown on *E. coli* HT115 until the young adult stage, followed by incubation on *P. aeruginosa* for 5 h (A) or 24 h (B). ***p < 0.001, **p < 0.01, and *p < 0.05 via the t test. n.s., non-significant.

(C) Gene expression analysis of N2 animals grown on *nol-6* RNAi bacteria until the young adult stage, followed by incubation on *P. aeruginosa* for 24 h. n.s., non-significant.

(D–H) Relative mRNA levels of *daf-7* (D), *flp-18* (E), *flp-21* (F), *npr-1* (G), and *tph-1* (H) in animals grown on RNAi control bacteria, as well as bacteria expressing RNAi against *aex-5*, *flr-1*, *nhx-2*, and *pbo-1*. ***p < 0.001, **p < 0.01, and *p < 0.05 via the t test. n.s., non-significant.

(I) Gene expression analysis of N2 animals grown on live *E. coli* until the young adult stage, followed by incubation on HK *E. coli* for 24 h at 20°C. The control animals were maintained on live *E. coli*. ***p < 0.001, and *p < 0.05 via the t test.

The activation of neuroendocrine pathway genes by *P. aeruginosa* colonization could be a consequence of either an increased load of the pathogen or bloating of the intestinal lumen. To distinguish these possibilities, we studied the changes in gene expression in the DMP-defective animals in the presence of *E. coli*. We reasoned that if the *P. aeruginosa* load is required for the induction of gene expression, then no changes would be expected in animals with bloated intestinal lumens on *E. coli*. However, if bloating of the intestinal lumen rather than the pathogen load induces gene expression, then the changes should be observed in the animals with bloated intestines, even in the absence of *P. aeruginosa*. The animals with bloated intestinal lumens on *E. coli* had significant induction of the neuroendocrine pathway genes compared with the control animals (Figures 5D–5H). Animals fed HK *E. coli*, which causes luminal bloating,

also exhibited significantly higher levels of mRNAs for the neuroendocrine pathway genes compared with control animals grown on live *E. coli* (Figure 5I). These results indicate that bloating of the intestinal lumen triggers a "flight" response in the animals through the induction of different neuroendocrine pathways that are required for pathogen avoidance.

The NPR-1 Neuroendocrine Pathway Is Required for Pathogen Avoidance Induced by Intestinal Bloating

Next, we assessed the neuroendocrine signaling pathway(s) that is required for the pathogen aversive behavior induced by bacterial colonization and bloating of the intestinal lumen. We inactivated genes required for the DMP in the loss-of-function mutants *daf-7*, *npr-1*, and *tph-1*, which are deficient in DAF-7, NPR-1, and serotonin signaling, respectively. While the
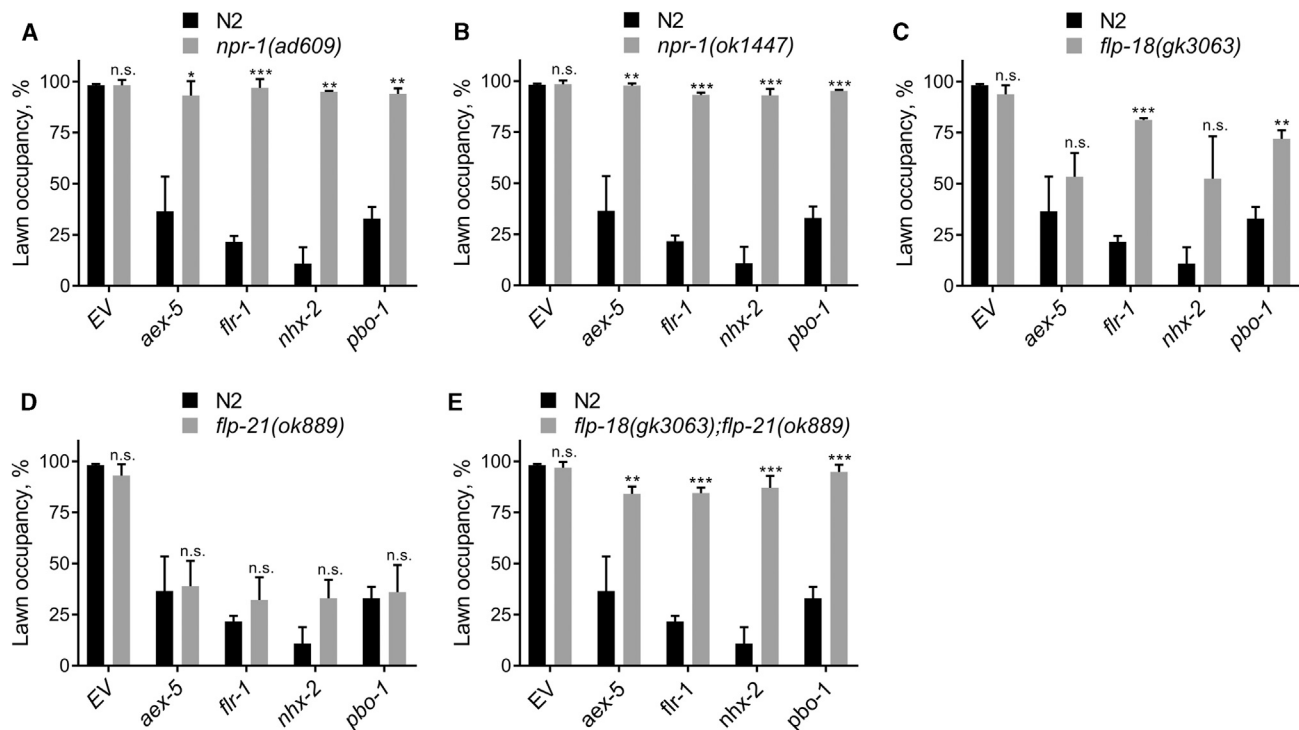
Figure 6. The NPR-1 Neural Pathway Is Required for Pathogen Avoidance Induced by Intestinal Lumen Bloating
(A–E) Percent lawn occupancy after 12 h of incubation on *P. aeruginosa* of *npr-1(ad609)* (A), *npr-1(ok1447)* (B), *flp-18(gk3063)* (C), *flp-21(ok889)* (D), and *flp-18(gk3063);flp-21(ok889)* (E) animals compared to control wild-type N2 animals. ***p < 0.001, **p < 0.01, and *p < 0.05 via the t test. n.s., non-significant. See also Figures S6 and S7.

tph-1(mg280) animals did not show any significant differences in avoidance compared to the wild-type N2 animals, *daf-7(e1372)* animals showed a partial, but significant, suppression of the enhanced *P. aeruginosa* avoidance behavior (Figure S6). In contrast, *npr-1(ad609)* animals showed complete suppression of the enhanced *P. aeruginosa* avoidance behavior of animals with bloated intestinal lumen (Figure 6A). This suppression is not due to the lack of bacterial colonization as the inhibition of DMP genes in *npr-1(ad609)* animals indeed led to enhanced bacterial colonization (Figure S7A and S7B). Another allele of *npr-1*, *npr-1(ok1447)*, also led to complete suppression of the enhanced *P. aeruginosa* avoidance behavior of the DMP-defective animals (Figure 6B).

The FMRF-like peptide FLP-18 is expressed in several neurons, and FLP-21 is expressed in both neurons and intestine (Rogers et al., 2003). These peptides play important roles in behaviors associated with NPR-1, such as behavioral quiescence and social feeding (Choi et al., 2013; Rogers et al., 2003). However, their role in pathogen avoidance behavior has not been studied to date. As shown in Figures 6C and 6D, while loss-of-function mutants of *flp-18* showed partial suppression of the enhanced pathogen avoidance of the DMP-defective animals, *flp-21* mutants fully retained the enhanced ability to avoid *P. aeruginosa*. However, *flp-18;flp-21* double mutant animals showed complete suppression of the enhanced avoidance of *P. aeruginosa* (Figure 6E), suggesting that they might act redundantly. This suppression of the avoidance behavior was not due to the lack of bacterial colonization (Figures S7C and S7D).

Taken together, these results suggest that DAF-7 and NPR-1 and its ligands play a role in elicitation of the pathogen avoidance behavior upon microbial colonization and bloating of *C. elegans* intestinal lumen.

DISCUSSION

Despite being a simple organism, *C. elegans* has evolved complex mechanisms to recognize pathogens as such and to respond to their presence by eliciting avoidance behavioral responses and innate immune pathways. Here, we show that intestinal lumen bloating induced by microbial colonization activates both immune pathways with microbicidal function and a microbial aversion behavior. We demonstrate that bloating activates different neuroendocrine signaling pathways and that the increased aversion behavior requires the DAF-7/TGF-β and NPR-1 pathways. We propose a model wherein bloating of the intestinal lumen caused by microbial colonization may be perceived as a danger signal that activates an immune fight-and-flight response or BIFF response (Figure 7). The results suggest that inputs from the intestine can aid in the recognition of a broad range of microbes and modulate host behavior via neuroendocrine signaling.

Bloating of the intestine leads to reduced levels of intestinal fat, which may result in a nutritional deficit in *C. elegans* (Sheng et al., 2015). Because poor nutrition may lead to food avoidance (Shtonda and Avery, 2006), it is possible that the avoidance behavior is elicited by reduced levels of intestinal fat in bloated
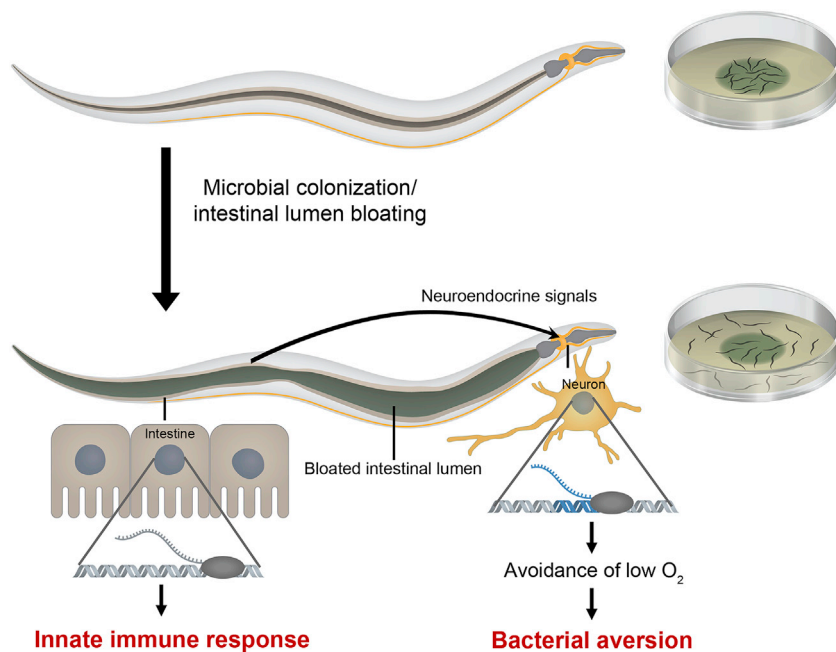
Figure 7. Model for the Microbial Colonization-Induced Immune Fight-and-Flight Response
Bloating of the intestinal lumen caused by microbial colonization activates a fight-and-flight response that results in the activation of innate immunity and a bacterial avoidance behavior. Bloating results in the upregulation of genes encoding the neuropeptide receptor NPR-1, its ligands, and TGF-β/DAF-7, which may promote bacterial avoidance by eliciting avoidance to low oxygen.

animals. However, *eat-5(ad1402)* and *eat-7(ad450)* animals do not exhibit intestinal bloating or pathogen avoidance behavior, despite having starvation phenotypes (Avery, 1993; Raizen et al., 2006), indicating that the nutritional status of the intestine is unlikely to mediate the bloating-induced avoidance behavior. Another possible mechanism by which intestinal bloating may elicit the avoidance behavior is through the mechanosensation of changes in the intestine and/or hypodermis. While the exact nature of the signaling mechanism that communicates the intestine with the nervous system is not understood, our data indicate that bloating induced by bacterial colonization might act as a "danger signal" that elicits the pathogen avoidance behavior.

In support of the idea that the physiological alterations caused by bloating of the intestinal lumen result in the induction of innate immune genes, earlier studies have shown that *C. elegans* mounts innate immune responses when core cellular activities are disrupted (Dunbar et al., 2012; McEwan et al., 2012; Melo and Ruvkun, 2012). RNAi and toxin-mediated disruption of core cellular activities, including translation, respiration, and protein turnover, induce the expression of detoxification and innate immune effectors (Melo and Ruvkun, 2012). Similarly, inhibition of *C. elegans* translation by *P. aeruginosa* exotoxin A triggers an innate immune response (Dunbar et al., 2012; McEwan et al., 2012). Together with these earlier findings, our study highlights the ability of *C. elegans* to sense physiological changes induced by pathogen infection or toxins and, in turn, induce an innate immune response. With such capabilities, the animals would be equipped to recognize a broader range of pathogens and toxins and mount defense responses.

One of our most surprising findings is the activation of neuroendocrine pathway gene expression by intestinal lumen bloating. This activation was only observed post-colonization of the intestine by pathogens and not at early time points of exposure, suggesting that the modulation of neuroendocrine signaling by pathogen exposure was triggered by bloating and not by simple exposure to the pathogen. Activation of the neuroendocrine signaling in animals with bloated intestinal lumens on live or HK *E. coli* further supports the conclusion that neuroendocrine signaling was modulated by bloating of the intestinal lumen and not by pathogen exposure. While the nervous system is known to control the physiology and behavior of animals by modulating the functionality of other tissues (Durieux et al., 2011; Prahlad et al., 2008; Reddy et al., 2009; Styer et al., 2008; Sun et al., 2011), the modulation of neural functions by non-neuronal cells to control physiology and behaviors remains understudied. Recent studies have shown that the intestine of *C. elegans* can modulate the nervous system (Chikka et al., 2016; Lee and Mylonakis, 2017), but the role of intestinal lumen bloating in this regulation has not been previously explored.

Intestinal lumen bloating was found to activate the expression of neuroendocrine pathway genes, including those that encode NPR-1/GPCR, DAF-7/TGF-β, and TPH-1, which is required for serotonin signaling. While the serotonin pathway was not found to be required for the elicitation of pathogen avoidance, it has been shown to be important for aversion of non-pathogenic *E. coli* when core cellular activities are disrupted (Melo and Ruvkun, 2012). Inhibition of the DAF-7/TGF-β pathway partially suppressed the enhanced pathogen avoidance behavior of animals with bloated intestinal lumens, whereas inhibition of NPR-1 caused complete suppression. Both of these pathways are important for the control of an aerotaxis behavior exhibited by *C. elegans* that is likely to impact microbial aversion (Meisel and Kim, 2014; Reddy et al., 2009, 2011; Styer et al., 2008). Inhibition of the NPR-1 and DAF-7/TGF-β pathways elicit avoidance of high oxygen, whereas higher activity of these pathways induces avoidance of low oxygen (De Bono and Bargmann, 1998; Chang et al., 2006; Meisel et al., 2014; Rogers et al., 2003). Therefore, the higher expression of *npr-1* and *daf-7* induced by bloating may elicit *C. elegans* avoidance of low oxygen, resulting in the avoidance of bacterial lawns, as they have low oxygen because of microbial metabolism (Figure 7). Consistent with this idea, the avoidance of *E. coli* lawns of DMP-defective animals is not as strong as that of *P. aeruginosa* lawns (Figure 3), which have lower oxygen levels than *E. coli* lawns (Reddy et al., 2011).

Our study suggests that bloating of the intestine may be a general mechanism involved in the recognition of a broad range of microbes and modulates host behavior via neuroendocrine

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signaling. In the future, it will be important to understand how intestinal bloating modulates neuroendocrine signaling. NPR-1 is closely related to the mammalian neuropeptide Y (NPY) receptors that are involved in the control of a diverse set of behavioral processes, including appetite, circadian rhythm, and anxiety (Heilig, 2004; Vona-Davis and McFadden, 2007; Yulyaningsih et al., 2011). Therefore, the identity and mode of action of the cues that are used by the intestine to activate the NPR-1 pathway may provide broad insights into the modulation of NPY-receptor-related behaviors such as appetite and anxiety.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at https://doi.org/10.1016/j.devcel.2019.02.001.

AUTHOR CONTRIBUTIONS

J.S. and A.A. conceived and designed the experiments. J.S. performed the experiments. J.S. and A.A. analyzed the data and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Escherichia coli OP50	*Caenorhabditis* Genetics Center (CGC)	OP50
Pseudomonas aeruginosa PA14	Laboratory of Frederick M. Ausubel	PA14
P. aeruginosa PA14-GFP	Laboratory of Frederick M. Ausubel	PA14-GFP
P. aeruginosa PA14 *gacA*	Laboratory of Meta Kuehn	PA14 *gacA*
Serratia marcescens Db11	CGC	Db11
Salmonella enterica serovar Typhimurium 1344	Laboratory of Frederick M. Ausubel	Typhimurium 1344
Enterococcus faecalis OG1RF	Laboratory of Danielle A. Garsin	OG1RF
Enterococcus faecium E007	Laboratory of Danielle A. Garsin	E007
Staphylococcus aureus NCTC8325	National Collection of Type Cultures	NCTC8325
E. coli HT115 (D3) containing plasmid pL4440	Source BioScience	HT115 L4440
Ahringer RNAi Libraries in *E. coli* HT115 (D3)	Source BioScience	N/A
Experimental Models: Organisms/Strains		
C. elegans: N2 wild-type	CGC	N2 Bristol
C. elegans: *npr-1(ad609)*	CGC	DA609
C. elegans: *npr-1(ok1447)*	CGC	RB1330
C. elegans: *flp-18(gk3063)*	CGC	VC2016
C. elegans: *flp-21(ok889)*	CGC	RB982
C. elegans: *flp-18(gk3063);flp-21(ok889)*	This study	N/A
C. elegans: *daf-7(e1372)*	CGC	CB1372
C. elegans: *tph-1(mg280)*	CGC	MT15434
C. elegans: *agIs26[myo-2p::mCherry,clec-60p::gfp]*	Laboratory of Javier E. Irazoqui	AU185
C. elegans: *phm-2(ad597)*	CGC	DA597
C. elegans: *eat-2(ad465)*	CGC	DA465
C. elegans: *eat-4(ad819)*	CGC	DA819
C. elegans: *eat-5(ad1402)*	CGC	DA1402
C. elegans: *eat-7(ad450)*	CGC	DA521
C. elegans: *eat-17(ok3041)*	CGC	RB2247
C. elegans: *eat-18(ad820)*	CGC	DA820
C. elegans: *egl-8(n488)*	CGC	MT1083
C. elegans: *rab-6.2(ok2254)*	CGC	VC2117
C. elegans: *unc-25(e156)*	CGC	CB156
C. elegans: *unc-47(e307)*	CGC	CB307
Oligonucleotides		
See Table S1 for primers used in the study	Integrated DNA Technologies	N/A
Recombinant DNA		
pbo-1 RNAi clone	This study	N/A
Software and Algorithms		
GraphPad Prism 7	GraphPad Software	https://www.graphpad.com/scientificsoftware/prism/
Photoshop CS5	Adobe	https://www.adobe.com/products/photoshop.html

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Alejandro Aballay (aballay@ohsu.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial Strains

The following bacterial strains were used: *Escherichia coli* OP50, *E. coli* HT115(DE3), *Pseudomonas aeruginosa* PA14, *P. aeruginosa* PA14 *gacA* mutant, *P. aeruginosa* PA14-GFP, *Serratia marcescens* Db11, *Salmonella enterica* serovar Typhimurium 1344, *Enterococcus faecalis* OG1RF, *Enterococcus faecium* E007, and *Staphylococcus aureus* NCTC8325. The *E. coli* OP50, *E. coli* HT115(DE3), *P. aeruginosa* PA14 strains, *S. marcescens*, and *S. enterica* cultures were grown in Luria-Bertani (LB) broth at 37°C. The *E. faecalis* and *E. faecium* cultures were grown in brain heart infusion (BHI) broth at 37°C. The *S. aureus* cultures were grown in tryptic soy (TS) broth at 37°C.

C. elegans Strains and Growth Conditions

C. elegans hermaphrodites were maintained on *E. coli* OP50 at 20°C unless otherwise indicated. Bristol N2 was used as the wild-type control unless otherwise indicated. Strains DA609 *npr-1(ad609)*, RB1330 *npr-1(ok1447)*, VC2016 *flp-18(gk3063)*, RB982 *flp-21(ok889)*, CB1372 *daf-7(e1372)*, MT15434 *tph-1(mg280)*, DA597 *phm-2(ad597)*, DA465 *eat-2(ad465)*, DA819 *eat-4(ad819)*, DA1402 *eat-5(ad1402)*, DA521 *eat-7(ad450)*, RB2247 *eat-17(ok3041)*, DA820 *eat-18(ad820)*, MT1083 *egl-8(n488)*, VC2117 *rab-6.2(ok2254)*, CB156 *unc-25(e156)*, and CB307 *unc-47(e307)* were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN). Strain AU185 *agIs26[myo-2p::mCherry,clec-60p::gfp]* was provided by Javier E. Irazoqui (University of Massachusetts Medical School, Worcester, MA 01605). The *flp-18*(gk3063);*flp-21*(ok889) double mutant was obtained by standard genetic crosses.

METHOD DETAILS

RNA Interference (RNAi)

RNAi was used to generate loss-of-function RNAi phenotypes by feeding nematodes *E. coli* strain HT115(DE3) expressing double-stranded RNA (dsRNA) homologous to a target gene (Fraser et al., 2000; Timmons and Fire, 1998). RNAi was carried out as described previously (Singh and Aballay, 2017). Briefly, *E. coli* with the appropriate vectors were grown in LB broth containing ampicillin (100 μg/mL) and tetracycline (12.5 μg/mL) at 37°C overnight and plated onto NGM plates containing 100 μg/mL ampicillin and 3 mM isopropyl β-D-thiogalactoside (IPTG) (RNAi plates). RNAi-expressing bacteria were allowed to grow overnight at 37°C. Gravid adults were transferred to RNAi-expressing bacterial lawns and allowed to lay eggs for 2 hours. The gravid adults were removed, and the eggs were allowed to develop at 20°C to young adults for subsequent assays. *unc-22* RNAi was included as a positive control to account for the RNAi efficiency. All RNAi clones except *pbo-1* were from the Ahringer RNAi library.

Construction of the *pbo-1* RNAi Clone

The 591-base-pair full-length cDNA of *pbo-1* was amplified using the forward primer 5'-GCTCCCGGGATGGGCCAAAATTCGTCTCA-3' and the reverse primer 5'-AGGTCTAGATGTCAAAAAACGGAAGGACATC-3'. The fragment was cloned into the SmaI and XbaI sites of pL4440 (Open Biosystems) and transformed into *E. coli* HT115(DE3) cells.

Avoidance Assay on Pathogens

The bacterial lawns were prepared by inoculating individual bacterial colonies into 2 mL of the corresponding broth mentioned above and growing them for 8-10 hours on a shaker at 37°C. Then, 20 μL of the culture was plated onto the center of a 3.5-cm plate and incubated at 37°C for 12-16 hours except for *S. aureus* which was incubated for 6 hours. For *P. aeruginosa* PA14 strains, *S. marcescens* and *S. enterica* modified NGM (3.5% instead of 2.5% peptone) plates were used. For *E. faecalis* and *E. faecium*, BHI agar plates were used. For *S. aureus*, TS agar plates were used. Thirty synchronized young gravid adult hermaphroditic animals grown on *E. coli* HT115(DE3) containing control vector or an RNAi clone targeting a gene were transferred outside the bacterial lawns, and the numbers of animals on and off the lawns were counted at the indicated times for each experiment. Three 3.5-cm plates were used per trial in every experiment. Experiments were performed at 25°C. The percent occupancy was calculated as $(N_{on}\ lawn/N_{total}) \times 100$. At least three independent experiments were performed.

Avoidance Assay on *E. coli*

Saturated overnight cultures of the *E. coli* HT115 RNAi bacteria were concentrated 30–40 times in LB. A 50-μL aliquot was plated onto the center of a 3.5-cm RNAi plate and incubated at 37°C overnight. The following day, the plates were cooled at room temperature for 1 hour, and then gravid adults were transferred to bacterial lawns and allowed to lay eggs for 2 hours. The gravid adults were removed, and the eggs were allowed to develop at 20°C. After 72 hours of incubation at 20°C, the numbers of animals on and off the lawns were counted to calculate aversion as $N_{off}\ lawn/N_{total}$. Three independent experiments were performed.

P. aeruginosa-GFP Colonization Assay

Bacterial lawns were prepared by inoculating individual bacterial colonies into 2 mL of LB with 50 μg/mL kanamycin and growing them for 8-10 hours on a shaker at 37°C. For the colonization assays, bacterial lawns of *P. aeruginosa*-GFP were prepared by spreading 20 μL of the culture on the complete surface of 3.5-cm-diameter modified NGM agar plates (0.35% instead of 0.25%

peptone). For *P. aeruginosa*-GFP colonization assays on partial lawns, bacterial lawns were prepared by placing 20 μL of the culture at the center of 3.5-cm-diameter modified NGM agar plates. The plates were incubated at 37°C for 12-16 hours and then cooled to room temperature for at least 1 h before seeding with young gravid adult hermaphroditic animals. The assays were performed at 25°C. At the indicated times for each experiment, the animals were transferred from *P. aeruginosa*-GFP plates to fresh *E. coli* OP50 plates and visualized within 5 minutes under a fluorescence microscope.

Quantification of Intestinal Bacterial Loads
P. aeruginosa-GFP lawns were prepared as described above. For quantification of colony forming units (CFU) at 5 hours of exposure, bacterial lawns of *P. aeruginosa*-GFP were prepared by spreading 20 μL of the culture on the complete surface of 3.5-cm-diameter modified NGM agar plates (0.35% instead of 0.25% peptone). For quantification of CFU on partial lawns, bacterial lawns were prepared by placing 20 μL of the culture at the center of 3.5-cm-diameter modified NGM agar plates. The plates were incubated at 37°C for 12-16 hours and then cooled to room temperature for at least 1 hour before seeding with young gravid adult hermaphroditic animals. The assays were performed at 25°C. At the indicated times for each experiment, the animals were transferred from *P. aeruginosa*-GFP plates to the center of fresh *E. coli* plates for 10 min to eliminate *P. aeruginosa*-GFP stuck to their body. Animals were transferred to the center of a new *E. coli* plate for 10 min to further eliminate external *P. aeruginosa*-GFP. Animals were transferred to fresh *E. coli* plates a third time for 10 min. Afterward, ten animals/condition were transferred into 50 μL of PBS plus 0.01% Triton X-100 and ground. Serial dilutions of the lysates (10^1, 10^2, 10^3, 10^4) were seeded onto LB plates containing 50 μg/mL of kanamycin to select for *P. aeruginosa*-GFP cells and grown overnight at 37°C. Single colonies were counted the next day and represented as the number of bacterial cells or CFU per animal. Three independent experiments were performed for each condition.

Fluorescence Imaging
Fluorescence imaging was carried out as described previously (Singh and Aballay, 2017). Briefly, animals were anesthetized using an M9 salt solution containing 30 mM sodium azide and mounted onto 2% agar pads. The animals were then visualized using a Leica M165 FC fluorescence stereomicroscope.

S. enterica Colonization Followed by *P. aeruginosa* Avoidance
Wild-type N2 L4 animals grown on *E. coli* HT115 were transferred to the lawns of *S. enterica*, followed by incubation at 25°C. Control animals on *E. coli* HT115 were also incubated at 25°C. After 48 hours of incubation, *S. enterica*-colonized animals, along with the animals grown on *E. coli* HT115, were transferred to *P. aeruginosa* lawns, and the numbers of animals on and off the lawns at the indicated times were counted. The experiments were performed at 25°C. The percent occupancy was calculated as ($N_{on\ lawn}/N_{total}$)×100. Three independent experiments were performed.

Cultivation of *C. elegans* on Heat-Killed *E. coli* OP50
A single colony of *E. coli* OP50 was inoculated in 100 mL of LB broth in a 500 mL conical flask and incubated at 37°C at 225 rpm shaking for 24 hours. Bacteria were then concentrated 10 to 20 times and heat-killed at 100°C for 1 hour. Bacterial death was confirmed by failure to grow on LB plates at 37°C overnight. The heat-killed bacteria were seeded on NGM plates containing 50 μg/mL of kanamycin and 100 μg/mL of streptomycin. Young adult wild-type N2 animals grown on *E. coli* HT115 RNAi control plates were washed with M9 medium and incubated at room temperature for 1 hour with M9 medium containing 50 μg/mL of kanamycin to remove live bacteria from their intestinal lumen. The animals were then washed with M9 medium and transferred to NGM plates containing heat-killed *E. coli* OP50 and incubated at 20°C for 24 hours.

Pharyngeal Pumping Assay
Wild-type N2 animals grown on heat-killed *E. coli* for 24 hours at 20°C were used for pharyngeal pumping assay with animals grown on live *E. coli* as controls. The number of contractions of the terminal bulb was counted over 1 minute. A contraction was defined as the backward movement of the grinder in the terminal bulb of the pharynx. The pumping rates for 20 nematodes were recorded for each condition.

Defecation Assay
Wild-type N2 animals grown on heat-killed *E. coli* for 24 hours at 20°C were used for defecation assay with animals grown on live *E. coli* as controls. The DMP cycle length was scored by assessing the time between expulsions (which are preceded by posterior and anterior body wall muscle contraction, and the contraction of enteric muscles in a normal regular pattern) (Thomas, 1990). Ten cycles each were measured for six different animals per condition.

RNA Isolation and Quantitative Reverse Transcription-PCR (qRT-PCR)
Animals were synchronized by egg laying. Approximately 35 N2 gravid adult animals were transferred to 10-cm RNAi plates seeded with *E. coli* HT115 expressing the appropriate vectors and allowed to lay eggs for 4 hours. The gravid adults were then removed, and the eggs were allowed to develop at 20°C. For the RNAi against the DMP genes, the animals were grown until they were one-day-old adults at 20°C. For the *P. aeruginosa* infection assays, the animals were first grown on 10-cm RNAi plates seeded with *E. coli* HT115 expressing either the empty vector RNAi control or *nol-6* RNAi until the young adult stage. Subsequently, the animals were collected,

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washed with M9 buffer, and transferred to 10-cm modified NGM plates (0.35% instead of 0.25% peptone) seeded with 300 μL of *P. aeruginosa* culture grown overnight. The *P. aeruginosa* plates were prepared by spreading 300 μL of the *P. aeruginosa* culture on the surface of the modified NGM plates, followed by an overnight incubation at 37°C. After transfer of the animals, the *P. aeruginosa* plates were incubated at 25°C for the indicated time points. Animals on the control *E. coli* plates were also incubated at 25°C. After the desired treatment (RNAi against the DMP genes, growth on heat-killed *E. coli* for 24 hours or *P. aeruginosa* infection), the animals were collected, washed with M9 buffer, and frozen in TRIzol reagent (Life Technologies, Carlsbad, CA). Total RNA was extracted using the RNeasy Plus Universal Kit (Qiagen, Netherlands). Residual genomic DNA was removed using TURBO DNase (Life Technologies, Carlsbad, CA). A total of 6 μg of total RNA was reverse-transcribed with random primers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

qRT-PCR was conducted using the Applied Biosystems One-Step Real-time PCR protocol using SYBR Green fluorescence (Applied Biosystems) on an Applied Biosystems 7900HT real-time PCR machine in 96-well-plate format. Twenty-five-microliter reactions were analyzed as outlined by the manufacturer (Applied Biosystems). The relative fold-changes of the transcripts were calculated using the comparative $CT(2^{-\Delta\Delta CT})$ method and normalized to pan-actin (*act-1, -3, -4*). The cycle thresholds of the amplification were determined using StepOnePlus software (Applied Biosystems). All samples were run in triplicate. The primer sequences are available in Table S1.

C. elegans Longevity Assays
Lifespan assays were performed on RNAi plates containing *E. coli* HT115(DE3) with the appropriate vector in the presence of 50 μg/mL of 5-fluorodeoxyuridine (FUdR). Animals were synchronized on RNAi plates without FUdR and incubated at 20°C. At the L4 larval stage, the animals were transferred onto the corresponding RNAi plates containing 50 μg/mL of FUdR. Animals were scored on a daily basis as alive, dead, or gone. Animals that failed to display touch-provoked movement were scored as dead. Experimental groups contained 60 to 100 animals. Young adult animals were considered as day 0 for the lifespan analysis. The assays were performed at 20°C. Three independent experiments were performed.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed with Prism 7 (GraphPad). All error bars represent the standard deviation (SD). The two-sample t test was used when needed, and the data were judged to be statistically significant when $p < 0.05$. In the figures, asterisks (*) denote statistical significance as follows: *, $p < 0.05$, **, $p < 0.001$, ***, $p < 0.0001$, as compared with the appropriate controls. The Kaplan-Meier method was used to calculate the survival fractions, and statistical significance between survival curves was determined using the log-rank test. For Figure S6, two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used. All experiments were performed in triplicate.

Supplemental Information

Microbial Colonization Activates

# an Immune Fight-and-Flight Response

via Neuroendocrine Signaling

Jogender Singh and Alejandro Aballay

Supplemental Information

Microbial colonization activates an immune fight-and-flight response via neuroendocrine signaling

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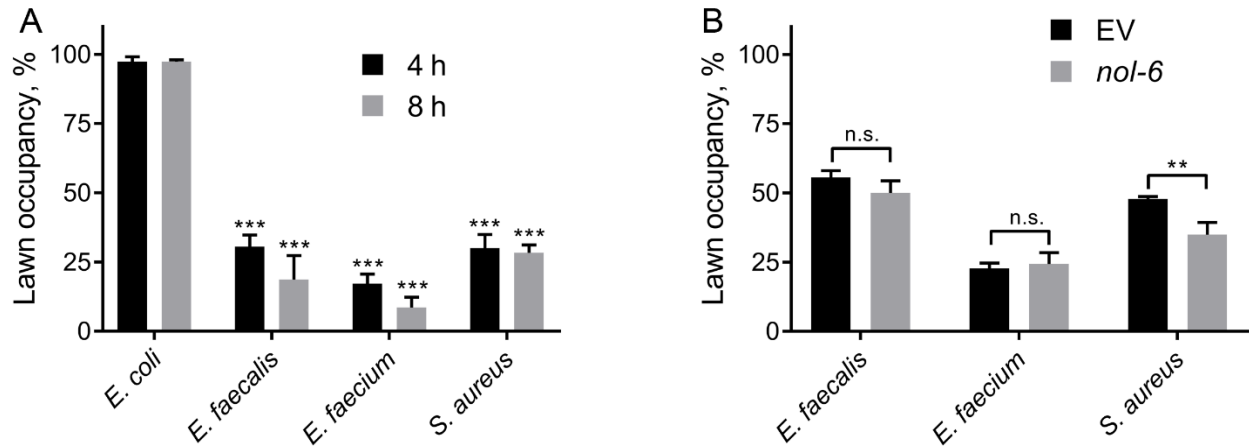
Figure S1. Microbial Pathogens Elicit Different Patterns of Avoidance in *C. elegans*, Related to Figures 1 and 2

(A) Occupancy of different bacterial lawns by wild-type N2 *C. elegans* at 4 hours and 8 hours after transfer to the lawns. p-values for *E. faecalis*, *E. faecium* and *S. aureus* are relative to *E. coli* for the corresponding time points. ***$p < 0.001$ via the t-test.

(B) Occupancy of different bacterial lawns by wild-type N2 animals grown on *nol-6* as well as RNAi control bacteria at 2 hours after transfer to the lawns. EV, empty vector RNAi control. **$p < 0.01$ via the t-test. n.s., non-significant.
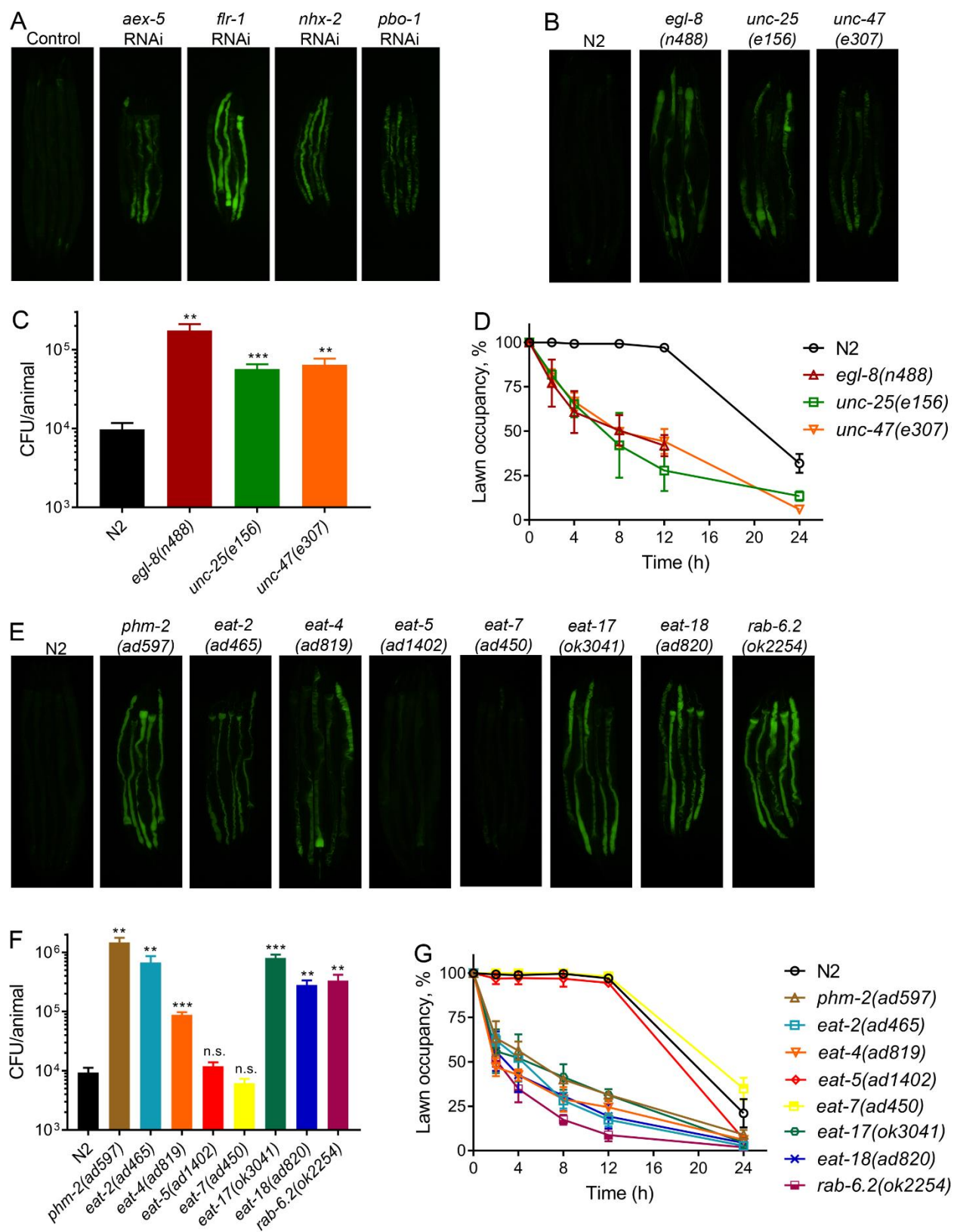
Figure S2. *P. aeruginosa* Colonization of the Intestine Induces Pathogen Avoidance, Related to Figure 3

(A) Representative photomicrographs of animals incubated on *P. aeruginosa*-GFP for 5 hours after growth on RNAi bacteria.

(B) Representative photomicrographs of young hermaphroditic adult N2, *egl-8(n488)*, *unc-25(e156)*, and *unc-47(e307)* animals incubated on *P. aeruginosa*-GFP for 5 hours at 25°C.

(C) Colony forming units per animal for young hermaphroditic adult N2, *egl-8(n488)*, *unc-25(e156)*, and *unc-47(e307)* animals exposed to *P. aeruginosa*-GFP for 5 hours at 25°C. ***p < 0.001, and **p < 0.01 via the t-test.

(D) Percent occupancy of *P. aeruginosa* lawns over time by N2, *egl-8(n488)*, *unc-25(e156)*, and *unc-47(e307)* animals.

(E) Representative photomicrographs of young hermaphroditic adult N2, *phm-2(ad597)*, *eat-2(ad465)*, *eat-4(ad819)*, *eat-5(ad1402)*, *eat-7(ad450)*, *eat-17(ok3041)*, *eat-18(ad820)*, and *rab-6.2(ok2254)* animals incubated on *P. aeruginosa*-GFP for 5 hours at 25°C.

(F) Colony forming units per animal for young hermaphroditic adult N2, *phm-2(ad597)*, *eat-2(ad465)*, *eat-4(ad819)*, *eat-5(ad1402)*, *eat-7(ad450)*, *eat-17(ok3041)*, *eat-18(ad820)*, and *rab-6.2(ok2254)* animals exposed to *P. aeruginosa*-GFP for 5 hours at 25°C. ***p < 0.001, and **p < 0.01 via the t-test. n.s., non-significant.

(G) Percent occupancy of *P. aeruginosa* lawns over time by N2, *phm-2(ad597)*, *eat-2(ad465)*, *eat-4(ad819)*, *eat-5(ad1402)*, *eat-7(ad450)*, *eat-17(ok3041)*, *eat-18(ad820)*, and *rab-6.2(ok2254)* animals.

A   *P. aeruginosa* WT   *P. aeruginosa gacA*

24 h

B

■ *P. aeruginosa* WT
■ *P. aeruginosa gacA*
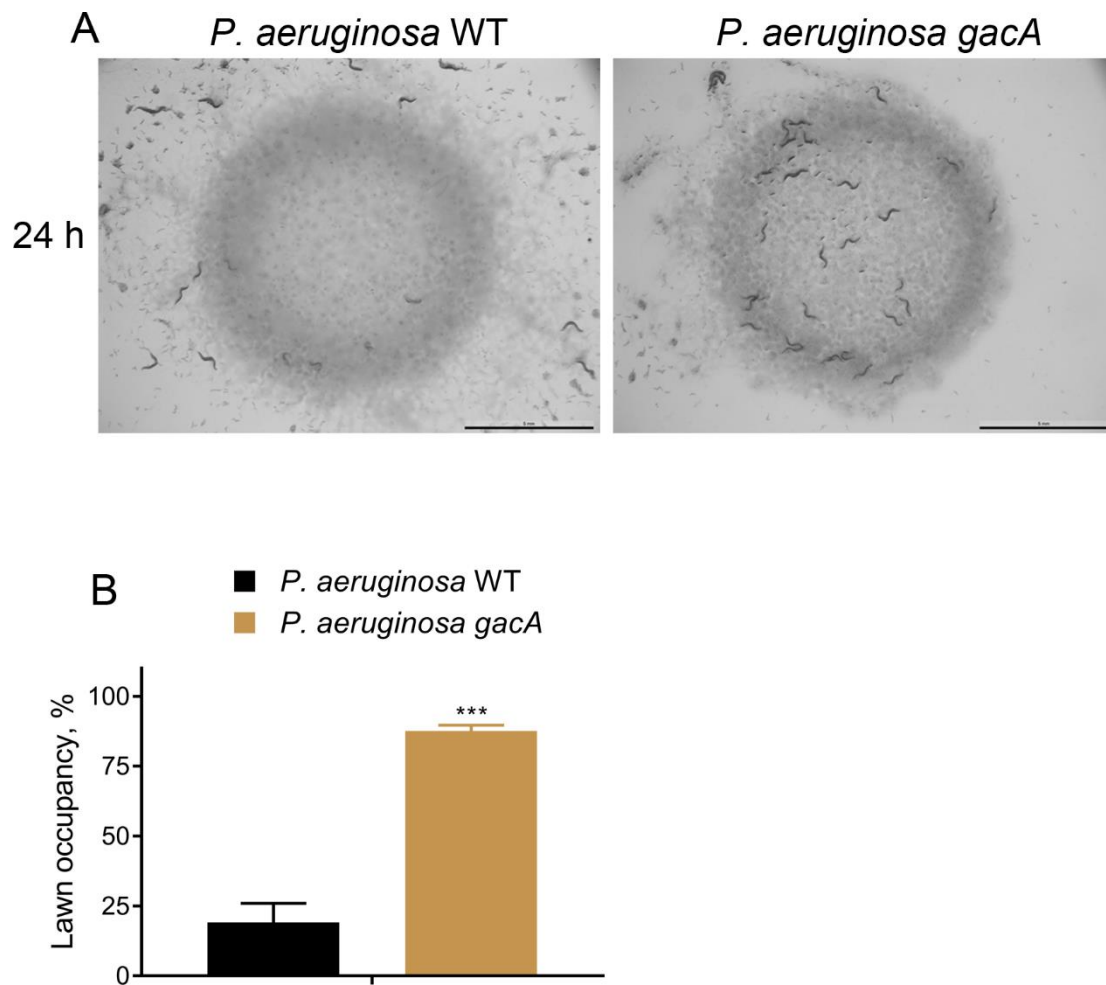
Figure S3. Avoidance Behavior of N2 animals on Different *P. aeruginosa* Variants, Related to Figure 3

(A) Representative photomicrographs of N2 animals on *P. aeruginosa* WT and *P. aeruginosa gacA* mutant after 24 hours of incubation.

(B) Percent occupancy of N2 animals on *P. aeruginosa* WT and the *P. aeruginosa gacA* mutant after 24 hours of incubation. ***$p < 0.001$ via the t-test.

N2
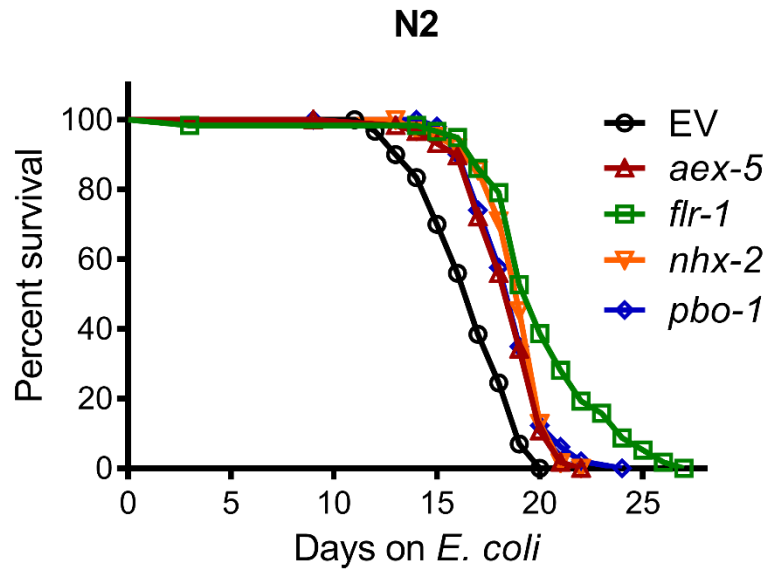
Figure S4. Intestinal Lumen Bloating Leads to an Increased Lifespan, Related to Figure 4

Survival of N2 animals grown on bacteria for RNAi against *aex-5*, *flr-1*, *nhx-2,* and *pbo-1* along with the empty vector control at 20°C. Day 0 represents young adults. The p values for *aex-5*, *flr-1*, *nhx-2,* and *pbo-1* RNAi relative to the values for the empty vector, EV control are ***p < 0.0001.
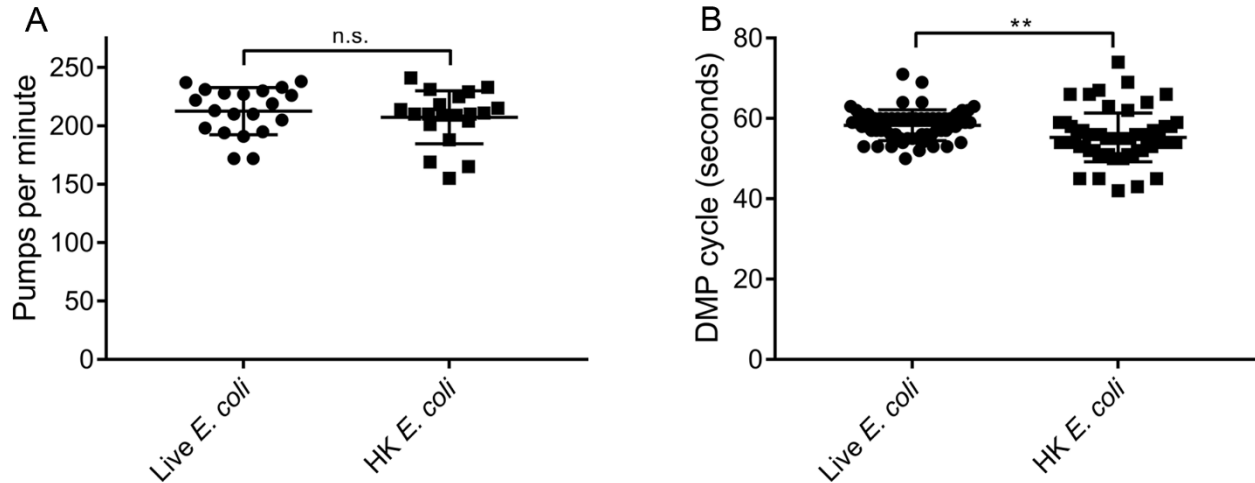
Figure S5. Pharyngeal Pumping Rate and DMP Cycle Length of Animals Fed Heat-Killed *E. coli*, Related to Figure 4

(A) Pharyngeal pumps per minute of N2 animals incubated on heat-killed (HK) *E. coli* for 24 hours at 20°C. The animals were grown on live *E. coli* until the young adult stage before incubation on HK *E. coli*. The control animals were maintained on live *E. coli*. n.s., non-significant.

(B) The DMP cycle length of N2 animals incubated on HK *E. coli* for 24 hours at 20 °C. The animals were grown on live *E. coli* until the young adult stage before incubation on HK *E. coli*. The control animals were maintained on live *E. coli*. **$p < 0.01$ via the t-test.
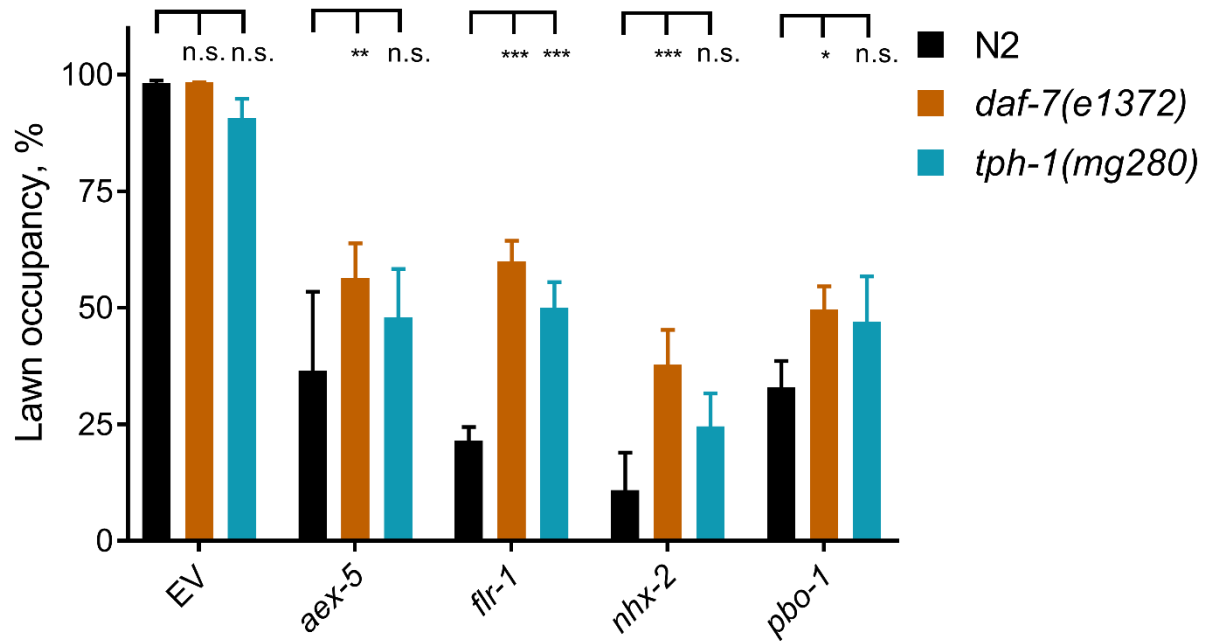
Figure S6. Role of the DAF-7 and TPH-1 Neural Pathways in Pathogen Avoidance Induced by Intestinal Bloating, Related to Figure 6

Percent lawn occupancy after 12 hours on *P. aeruginosa* of N2, *daf-7(e1372),* and *tph-1(mg280)* animals. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$. n.s., non-significant.
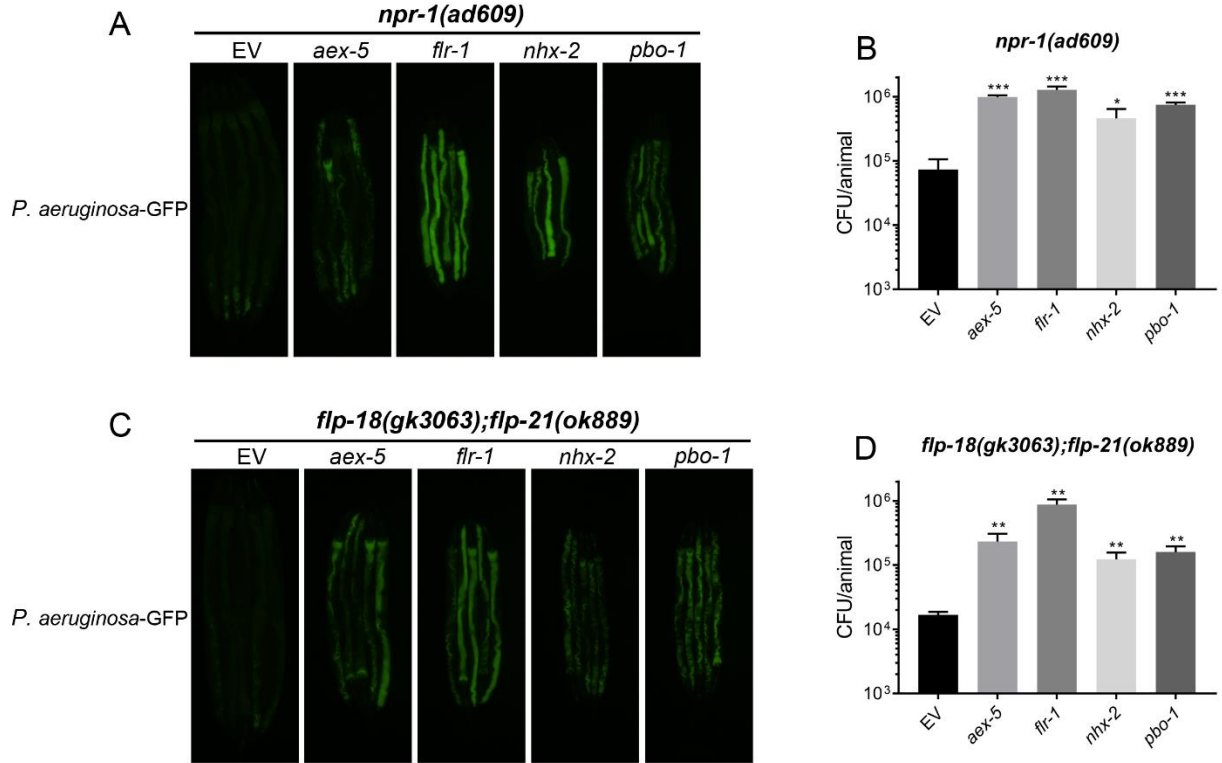
Figure S7. DMP Gene Knockdown Leads to Enhanced *P. aeruginosa* Colonization in *npr-1(ad609)* and *flp-18(gk3063);flp-21(ok889)* Animals, Related to Figure 6

(A) Representative photomicrographs of *npr-1(ad609)* animals exposed to *P. aeruginosa*-GFP for 5 hours at 25°C after growth on RNAi bacteria.

(B) Colony forming units per animal for *npr-1(ad609)* animals exposed to *P. aeruginosa*-GFP for 5 hours at 25°C after growth on RNAi bacteria. ***$p < 0.001$, and *$p < 0.05$ via the t-test.

(C) Representative photomicrographs of *flp-18(gk3063);flp-21(ok889)* animals exposed to *P. aeruginosa*-GFP for 5 hours at 25°C after growth on RNAi bacteria.

(D) Colony forming units per animal for *flp-18(gk3063);flp-21(ok889)* animals exposed to *P. aeruginosa*-GFP for 5 hours at 25°C after growth on RNAi bacteria. **$p < 0.01$ via the t-test.

Table S1. Primer used in the study, Related to Figures 4 and 5

Primer for quantitative reverse transcription-PCR		
Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pan-*act*	TCGGTATGGGACAGAAGGAC	CATCCCAGTTGGTGACGATA
clec-60	CTGCAGTCTGGATGGCAGAA	CGCAAGATGCCGTTTCACAA
cpr-2	ATCAAAGGGAGGACACGCTG	AAGAGTTGACAGCGAGCCAA
F53A9.8	CAAGAACACGGACACGGAGA	ATGTTGGGTGTCGCAGTGAT
ilys-3	AAGAGGTGCGCGAATGATCT	CCTGTCCAGTTCCAGCACAT
lys-4	CTAGAGCAGCTGGCCTCAC	GCAGCAGATGGGCAGTTAGA
lys-5	GCTCCATTCGGAGGATGGTC	GACTCCACACGGACCAGTAG
ugt-18	GGGTTGGGCAGTACAATGGA	ACTGAAGACATCCTCCGTGC
daf-7	GGGTGCTGCTTGTATGACCT	CCAAGTTGAAGTGGTGTGCG
flp-18	GTTCGACAAGCGCAGTGAAG	CCCTGCGTAATGCTCTTCCA
flp-21	GCGGCTGTTCATCTTGCTTT	TAAACTCTGTCACTGCCGGG
npr-1	ACTCTATGCGTGGCTGAACC	TCACTTTTACGCCGGTCTCC
tph-1	TCAGATGGCATCCGCAATGA	CCACTTCGACGAAAACGACG
Primer for construction of RNAi clone		
Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
pbo-1	GCTCCCGGGATGGGCCAAAATTCGTCTCA	AGGTCTAGATGTCAAAAAACGGAAGGACATC