Genetic Dissection of *Anopheles gambiae* Gut Epithelial Responses to *Serratia marcescens*

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Abstract

Genetic variation in the mosquito *Anopheles gambiae* profoundly influences its ability to transmit malaria. Mosquito gut bacteria are shown to influence the outcome of infections with *Plasmodium* parasites and are also thought to exert a strong drive on genetic variation through natural selection; however, a link between antibacterial effects and genetic variation is yet to emerge. Here, we combined SNP genotyping and expression profiling with phenotypic analyses of candidate genes by RNAi-mediated silencing and 454 pyrosequencing to investigate this intricate biological system. We identified 138 *An. gambiae* genes to be genetically associated with the outcome of *Serratia marcescens* infection, including the peptidoglycan recognition receptor PGRPLC that triggers activation of the antibacterial IMD/REL2 pathway and the epidermal growth factor receptor *EGFR*. Silencing of three genes encoding type III fibronectin domain proteins (*FN3D*) increased the *Serratia* load and altered the gut microbiota composition in favor of *Enterobacteriaceae*. These data suggest that natural genetic variation in immune-related genes can shape the bacterial population structure of the mosquito gut with high specificity. Importantly, *FN3D2* encodes a homolog of the hypervariable pattern recognition receptor Dscam, suggesting that pathogen-specific recognition may invoke a broader family of immune factors. Additionally, we showed that silencing the gene encoding the gustatory receptor Gr9 that is also associated with the *Serratia* infection phenotype drastically increased *Serratia* levels. The Gr9 antibacterial activity appears to be related to mosquito feeding behavior and to mostly rely on changes of neuropeptide F expression, together suggesting a behavioral immune response following *Serratia* infection. Our findings reveal that the mosquito response to oral *Serratia* infection comprises both an epithelial and a behavioral immune component.

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Introduction

Genetic variation within populations of the *An. gambiae* mosquito, especially with regard to genes encoding immune factors, is believed to play an important role in the mosquito susceptibility to infection by the malaria parasite *Plasmodium falciparum* [1–5]. Many immune factors exhibit both anti-*Plasmodium* and antibacterial activities, such as those involved in the IMD/REL2 pathway, which is triggered by bacteria through the peptidoglycan recognition receptor PGRPLC [4,5]. Bacterial infections can affect mosquito survival [6] and are thought to constitute a major evolutionary drive [7] as opposed to *Plasmodium* infections the impact of which on mosquito fitness is unclear [8]. An example is the segregation of *TEP1* alleles between the M and S molecular forms of *An. gambiae* in west Africa, which differentially affect *Plasmodium* infections, and is thought to be largely driven by bacterial pathogen pressure in larval habitats [2]. Therefore, genetic associations related to the outcome of bacterial infections may, directly or indirectly, influence mosquito vectorial capacity.

The adult mosquito gut harbors a wide spectrum of bacterial populations, mainly Gram-negative enterobacteria [9–11]. The broad variation in gut microbiota composition observed both at the individual and population levels is probably the result of an interplay between the environmental bacterial diversity and the mosquito genetic makeup [12–14]. Moreover, a precipitous bacterial increase after a blood meal, whose peak coincides with midgut invasion by *Plasmodium* [15], can affect the *Plasmodium* infection load both indirectly, by triggering PGRPLC-mediated mosquito immune responses [5,16] or through generation of immune memory [17], and directly, through the generation of reactive oxygen species by specific enterobacteria that compromise malaria parasites [18].

Epithelial responses against Gram-negative bacteria have been extensively studied in *Drosophila* [19]. They involve recognition of peptidoglycan [20,21] that triggers a finely-tuned immune response mainly through the *Imd* pathway, resulting in the expression of antimicrobial peptides that limit bacterial populations [22,23]. Production of reactive oxygen species, which target bacteria, through the Dual Oxidase (DUOX) pathway, has also been reported [24]. Gut stem cell proliferation and epithelial cell renewal following tissue damage due to bacterial infection are regulated by the EGFR and JAK/STAT pathways [25–27]. However, the mechanisms involved in achieving gut homeostasis
Author Summary

In malaria vector mosquitoes, the presence of bacteria and malaria parasites is tightly linked. Bacteria that are part of the mosquito gut ecosystem are critical modulators of the immune response elicited during infection with malaria parasites. Furthermore, responses against oral bacterial infections can affect malaria parasites. Here, we combined mosquito gut infections with the enterobacterium Serratia marcescens with genome-wide discovery and phenotypic analysis of genes involved in antibacterial responses to characterize molecular processes that control gut bacterial infections thus possibly affecting the mosquito susceptibility to infection by malaria parasites. Our data reveal complex genetic networks controlling the gut bacterial infection load and ecosystem homeostasis. These networks appear to exhibit much higher specificity toward specific classes of bacteria than previously thought and include behavioral response circuits involved in antibacterial immunity.

Results

S. marcescens infection of the mosquito gut

An. gambiae female adults were treated with antibiotics to reduce their natural gut microbiota load (Figure S1) and subsequently fed with fluorescently labeled S. marcescens (Db11-GFP) added to the sugar meal. The bacterial levels in the gut of sugar-fed mosquitoes (henceforth referred to as infection) were monitored from day 2 to 6 post infection and showed considerable variation including highly and lowly infected mosquitoes as well as mosquitoes that despite ingesting bacteria-containing sugar showed no sign of fluorescence in their gut (Figure 1A). While the proportion of lowly infected mosquitoes remained rather constant at approximately 50% throughout the course of the experiment, the relative proportions of highly and non-infected mosquitoes changed between days 2 and 3 in favor of highly infected mosquitoes and remained stable thereafter until day 5 (Figure 1B). At day 6, highly infected mosquitoes decreased by ca. 15% with a parallel increase of non-infected mosquitoes.

Identification of SNP divergence associated with the outcome of S. marcescens infection

To investigate whether genetic variation could partly explain the observed S. marcescens infection phenotype, single nucleotide polymorphism (SNP) divergence between the highly and non-infected phenotypic pools was interrogated using a 400 k SNP genotyping array. Mosquitoes were orally infected with S. marcescens, and gut infection levels were determined at day 5 post infection. The results were similar to those obtained in the previous replicate experiments: 38.4% of mosquitoes could be classified as highly infected, 48.6% lowly infected and 13% non-infected (Figure 1C). Pools of equimolar amounts of genomic DNA (gDNA) prepared from carcasses of 15 highly infected and 15 non-infected mosquitoes out of 139 and 47 mosquitoes in each phenotypic group, respectively, were hybridized onto two Affymetrix SNP genotyping arrays. These SNP chips interrogate genetic variation at ~400,000 variable positions in the An. gambiae genome (Table S1) [38], and were previously shown to provide useful quantitative information regarding divergence between pooled mosquito samples [39].

Allele calls for each SNP locus were used to determine the minor allele frequency (MAF) differences between highly and non-infected gDNA pools. Two approaches were used to assess genotypic association with the S. marcescens infection phenotype. The first included MAF difference at a SNP locus between highly infected and non-infected pools >0.5, suggesting a preponderance of different genotypes between the two pools for the respective locus. The second involved a permutation analysis in which the average MAF difference of 10 adjacent SNP loci (SNPs) was compared with that of 10 random SNPs. Statistical significance was assessed for each of the ~40,000 non-overlapping 10-SNP windows (Table S2) and those showing a p-value<10^-7, following a Bonferroni correction for the number of tests conducted, were considered as being associated with the S. marcescens infection phenotype.

The two approaches detected 140 SNPs with MAF difference >0.5 and 44 10-SNP windows with significant p-values, respectively. As shown in Figure 2, these SNPs and 10-SNP windows together formed distinctive clusters along the An. gambiae genome that were designated as peaks so that they are discerned from each other, although assessed association was limited to genes within a 5 kb radius of highlighted SNPs or within genomic areas delineated by significant 10-SNP windows. Overall, 118 genes were found to reside within a 5 kb radius of highlighted SNPs (Table S3), while 27 genes fell within significant 10-SNP windows (Table S4). The two approaches combined detected 138 genes (Table S5), as there was an overlap of 7 genes between the two sets, including the highly relevant CLIP6 and EGFR as discussed below.
In peak 2L-5 (chromosome 2L, peak 5), the gene encoding PGRPLC is found within a 5 kb radius of a highlighted SNP. PGRPLC recognizes peptidoglycan and activates the IMD/REL2 NF-κB signaling pathway, thus eliciting antibacterial responses [21,40,41]. This pathway is constitutively triggered by mosquito gut bacteria maintaining an elevated level of antimicrobial peptide production [5,42]. The association of PGRPLC with the S. marcescens infection phenotype suggests that genetic variation within the mosquito population may influence the ability to mount an antibacterial response via the IMD/REL2 pathway.

Adjacent to PGRPLC, in peak 2L-5, is another peptidoglycan recognition protein encoding gene, PGRPLA.

Of the remaining genes, several exhibit homologies suggesting involvement in antibacterial immune responses, especially in recognition of pathogen or host derived signals as well as in signal transduction and regulation of immune responses (Table 1). The permutation analysis revealed 3 genes, out of a total of 27, encoding proteins with type III fibronectin domains (FN3D) in different peaks: FN3D1 in peak 2L-4, which was also in the proximity of a highlighted SNP, FN3D2 in 2L-14 and FN3D3 in 2R-4. A total of 65 An. gambiae genes contain FN3 domains, including the hypervariable pattern recognition receptor AgDscam, the insulin receptor INR and the JAK/STAT receptor DOME. FN3D2 and FN3D3 additionally possess immunoglobulin and putative transmembrane domains, while FN3D2 is an ortholog of Drosophila Dscam4. Drosophila Dscam is shown to bind bacteria and influence the efficiency of phagocytosis [43], while its An. gambiae ortholog, AgDscam, is also shown to bind bacteria and mediate antibacterial and anti-Plasmodium responses [44]. Importantly, Dscam genes in various organisms generate a diverse repertoire of isoforms, suggestive of challenge-specific pattern recognition through alternative splicing [43,45,46], with particular AgDscam isoforms specifically targeting P. berghei, P. falciparum or commensal bacteria [47,48].

Several putative transcription factors with homeobox-like or DNA-binding domains were found in the identified peaks. AGAP005096 in 2L-4 and AGAP005244 in 2L-5 (together with PGRPLC and PGRPLA) encode homeodomains. The homeobox gene, Caudal, has been previously implicated in the regulation of epithelial immune responses and shown to influence the gut bacterial population structure in Drosophila [28], while its mosquito homolog has been shown to regulate the IMD/REL2 pathway.

Figure 1. Gut infection with S. marcescens varies between individual An. gambiae mosquitoes. Mosquitoes were antibiotic treated for 5 days and subsequently fed on sugar containing the Db11-GFP strain of S. marcescens. Bacteria-fed mosquitoes were selected 2 days post infection and the prevalence of fluorescent bacteria in their gut was monitored from day 2 to 6 post infection. 1A: The level of S. marcescens infection in the mosquito gut showed considerable variation: mosquitoes with intense fluorescence in most of the gut were characterized as highly infected (left panel), mosquitoes in which fluorescence was evident but confined to a part of the gut were characterized as lowly infected (middle panel) and mosquitoes with no sign of fluorescence were characterized as non-infected (right panel). 1B: S. marcescens infected mosquitoes were dissected each day, from day 2 to 6 post infection, and the proportions of highly, lowly and non-infected mosquitoes were determined over 4 independent infections. The average percentage ± SEM for each level of infection is indicated for each day post infection, with the total number of mosquitoes dissected each day in all 4 infections shown over each bar. 1C: In 2 independent infections used for SNP genotyping, mosquitoes were dissected 5 days post infection and the percentage of highly, lowly and non-infected mosquitoes, pooled from both infections, can be seen beside the respective part of the bar representing each level of infection.

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Thus, these putative transcription factors could play similar regulatory roles. AGAP002492 in peak 2R-7 encodes a DNA-binding domain, while its Drosophila ortholog, ewg, is involved in the Wnt/Wingless pathway [50]. AGAP005156, in peak 2L-4 encodes an ARID/BRIGHT DNA-binding domain, with its Drosophila ortholog, retained, is involved in behavioral modulations and repression of male courtship [51,52]. AGAP005661, in peak 2L-7, a putative ligand-regulated transcription factor, is an ortholog of the Drosophila nuclear receptor FTZ-F1, involved in juvenile hormone mediated gene expression [53].

Genes encoding alpha-glucosidase and alpha-mannosidase homologs were detected in peaks 2R-1 and 2R-13, respectively. These genes possess glycoside hydrolase domains that are also present in the conserved chitinase gene family [54], involved in bacterial clearance and host tolerance [55].

The gene encoding the epidermal growth factor receptor, EGFR, was identified in the prominent peak 3R-6 both by both the permutation and the individual SNP analysis. The Drosophila EGFR pathway has been implicated in gut remodeling following oral bacterial infection [27], suggesting that the EGFR pathway may influence the outcome of S. marcescens infection in Anopheles, possibly through synergistic functions in gut homeostasis.

CLIPE6 and CLIPE7, found in peak 3L-16, belong to the non-catalytic E sub-family of CLIP-type serine proteases, a family known to participate in proteolytic cascades in antibacterial and anti-Plasmodium responses [56,57], with SPCLIP1, another E subfamily member, involved in anti-Plasmodium responses by regulating complement recruitment [58,59]. Several leucine-rich repeat containing genes were also detected, including LRIM15 (peak 2L-13), a transmembrane member of the LRIM family of immune

Figure 2. Mapping of An. gambiae genetic variation associated with the S. marcescens infection phenotype. SNPs with MAF difference >0.5 and 10-SNP windows with Bonferroni-corrected significance (p-value < 10^-5) are shown in their respective chromosomal position as red X crosses and dots, respectively. Non-significant 10-SNP windows are shown as blue dots. Genomic areas with highlighted SNPs and/or significant 10-SNP windows in close proximity are referred to as peaks and are numbered. Each peak is referred to using the chromosomal arms it resides on and its respective assigned number. The genomic positions of genes of interest found within a 5 kb radius of highlighted SNPs or within genomic areas delineated by 10-SNP windows with a significant p-value are indicated by vertical arrows.

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proteins [60]. LRIMs have also been implicated in complement anti-Plasmodium responses [61–63].

Two Toll-like receptors, TOLL1A and a previously uncharacterized paralog of TOLL5B, were found in peak X-4. Little is known about the role of Toll-like receptors in Anopheles immunity, however, cross-talk between the REL1 and REL2 signaling pathways in the yellow fever mosquito Aedes aegypti [64] and synergistic interactions between the Toll and Imd pathway in Drosophila [65], leave open the possibility for involvement of Toll-like receptors in defenses against Gram-negative bacteria, also in Anopheles [66].

A gene encoding a protein with a ricin B lectin domain was found in peak 2R-15. Lectins bind oligosaccharides and have been shown to modulate mosquito immune responses [6,63], while mammalian lectins modulate host and gut microbiota interactions [67]. Genes belonging to other families of putative pattern recognition receptors were also found to be associated with the S. marcescens infection phenotype, including a fibrinogen-related protein (FBN or FREP) and a galectin in peak 3L-10 and an MD2-like receptor in 2L-16 [59,68,69].

Five annotated or putative GPCRs were found to be associated with the S. marcescens infection phenotype, including three putative neurotransmitter-triggered receptors: the serotonin receptor GPR5HT7 in peak 2R-14, the GABA-B family receptor GPRGBB1 in peak 3R-15 and the neuropeptide receptor GPRNPR2 in 3R-5.

GPCRs have been previously implicated in modulation of P. falciparum infection in An. gambiae [70], but the mechanism by which this is accomplished remains unclear. NPR-1, a neurotransmitter-triggered GPCR of Caenorhabditis elegans, has been shown to modulate antibacterial defenses in a behavior dependent or independent manner, and NPR-1 genetic polymorphisms are suggested to be major determinants of bacterial susceptibility [71,72]. Serotonin is a major modulator of mammalian intestinal inflammation [73,74], in an interplay between the nervous and immune system [75]. The Drosophila ortholog of GPR5HT7 is involved in various behavioral processes [76,77], including

### Table 1. Genes of interest associated with the S. marcescens infection phenotype.

| Gene ID     | Name/Description | SNP/Permutation analysis | Peak (Figure 2) |
|------------|------------------|--------------------------|-----------------|
| AGAP001111 | alpha-glucosidase| Permutation              | 2R-1            |
| AGAP004032 | alpha-mannosidase| SNP                      | 2R-13           |
| AGAP011785 | CLIP6            | SNP, Permutation         | 3L-16           |
| AGAP011786 | CLIP7            | SNP, Permutation         | 3L-16           |
| AGAP002492 | DNA-binding      | Permutation              | 2R-7            |
| AGAP005661 | DNA-binding      | SNP                      | 2L-7            |
| AGAP005156 | DNA-binding      | SNP                      | 2L-4            |
| AGAP008819 | EGFR             | SNP, Permutation         | 3R-6            |
| AGAP005147 | FN3D1            | SNP, Permutation         | 2L-4            |
| AGAP007092 | FN3D2            | Permutation              | 2L-14           |
| AGAP001824 | FN3D3            | Permutation              | 2R-4            |
| AGAP011277 | FREP6            | SNP                      | 3L-10           |
| AGAP011278 | GALE4            | SNP                      | 3L-10           |
| AGAP004223 | GPRSH17          | SNP                      | 2R-14           |
| AGAP010281 | GPRGBB1          | SNP                      | 3R-15           |
| AGAP008702 | GPRNP2           | SNP                      | 3R-5            |
| AGAP009804 | G10              | SNP                      | 3R-11           |
| AGAP009805 | G9               | SNP                      | 3R-11           |
| AGAP005096 | Homeobox         | Permutation              | 2L-4            |
| AGAP005244 | Homeodomain      | SNP                      | 2L-5            |
| AGAP007291 | IAP4             | SNP                      | 2L-15           |
| AGAP007292 | IAP5             | SNP                      | 2L-15           |
| AGAP007045 | LRIM15           | SNP                      | 2L-13           |
| AGAP007415 | ML12             | SNP                      | 2L-16           |
| AGAP005205 | PGRPLA           | SNP                      | 2L-5            |
| AGAP005203 | PGRPLC           | SNP                      | 2L-5            |
| AGAP012252 | Protein C kinase 33E | SNP                | 3L-19           |
| AGAP004375 | Ricin B lectin   | Permutation              | 2R-15           |
| AGAP001004 | TOLL1A           | SNP                      | X-4             |
| AGAP001002 | Toll-like receptor | SNP                   | X-4             |

The Gene ID is shown along with its assigned name, if any, or a homology description (Name/Description column). The SNP/Permutation analysis column indicates whether association is based on the presence of the gene within a 5 kb radius of a SNP with MAF difference >0.5 (SNP) or within a significant 10-SNP window (Permutation). The peak each gene is found corresponds to the designation shown in Figure 2.
aggressive behavior, a process also modulated by NPF [78]. Interestingly, the *Drosophila* ortholog of GPRGB1 has been implicated in behavioral responses to alcohol sensitivity [79], a process in which NPF is also a major modulator [80,81].

Two gustatory receptor genes, *Gr9* and *Gr10*, encoding 7-transmembrane chemoreceptor domains, were associated with the outcome of *S. marcescens* infection (Figure 2, peak 3R-11). *Gr9* and *Gr10* are paralogs and show co-orthologous relationships with the *Drosophila* *Gr32a*, *Gr39a* and *Gr60a* [92]. *Gr32a* and *Gr60a* act as pheromone receptors in modulating mating behavior [93,94], while *Gr39a* has been implicated, through 4 splice variants, in sustaining courtship behavior [85]. *Gr32a* is also involved in regulating aggressive behavior through recognition of small non-volatile hydrocarbons [86], or feeding suppression triggered by DEET or other antifeedants [87].

Gustatory receptor family members have also been implicated in aversive taste [35,88], CO₂ responses [89,90] and sugar recognition [91–94]. A *Drosophila* gustatory receptor, *Gr43a*, has been shown to recognize fructose and act as a nutrient sensor, promoting or suppressing feeding [34]. Since enhanced or suppressed feeding of bacteria-containing sugar can decisively influence the abundance of *S. marcescens* that the mosquito takes in and its immune system can handle, it is possible that *Gr9* or *Gr10* variants linked to altered mosquito feeding behavior can affect the outcome of infection. Furthermore, GPR43, a mammalian chemoattractant receptor, has been shown to recognize short-chain fatty acids of bacterial origin and participate in antibacterial responses [36], while other mammalian chemoattractant receptors regulate inflammatory responses by recognizing endogenous factors [95]. Recognition of bacterial-derived uracil has recently been shown to modulate *Drosophila* antibacterial responses through the DUOX pathway [29]. Therefore, another possibility is that *Gr9* or *Gr10* recognize bacterial-derived metabolites or infection-induced mosquito molecules and mediate antibacterial responses.

Several other genes with no known or unrelated to immune responses homologies were also associated with the *S. marcescens* infection phenotype such as AGAP013604 in peak 2R-8, encoding a putative miRNA. miRNAs are known to modulate gene regulation in processes that include epithelial immunity [96,97]. AGAP006405 in peak 2L-10 encodes a tyrosine protein kinase, while its *Drosophila* ortholog, *droske2*, is involved in Wnt5 signaling and establishment of olfactory circuits [98]. In peak 2L-13 the inhibitors of apoptosis IAP4 and IAP5 were found. The *Drosophila* IAP2 is known to regulate 1md signaling [99], suggesting that the *An. gambiae* IAP4 or IAP5 may also play similar roles. AGAP012252, in peak 3L-19, encodes the ortholog of *Drosophila* PKC59E, implicated in NPF-mediated alcohol sensitivity [100,101]. AGAP011363, in peak 3L-11, encodes the ortholog of *Drosophila* rab6, implicated in phagocytosis [102] but also trafficking of Grk, the EGFR ligand [103,104]. AGAP010503, in peak 3L-4, encodes the ortholog of *Drosophila* SK channel, implicated in behavioral courtship memory [105]. AGAP005216, in peak 2L-5, encodes the ortholog of *Drosophila* fab1, involved in autophagy but also the lysosomal degradation of necrotic, a modulator of the Toll pathway [106–109].

Candidate gene prioritization for further phenotypic analysis was based on homologies with genes known to be involved in species-specific antibacterial responses, e.g. FN3D2 and *Drosophila* *PKC59E* [43] or demonstrably regulating the response to gut microbiota in other systems, e.g. *Gr9* and the mammalian chemoattractant receptor *GPR43* [36], with the aim of the identification of novel functions of genes or gene families in antibacterial responses.

**Serratia** infection phenotypic analysis of *FN3D1-3*

The involvement of the three *FN3D* genes in shaping the outcome of *An. gambiae* gut infection with *S. marcescens* was investigated by RNAi-mediated gene silencing (Figure 3). Antibiotic treated mosquitoes were orally infected with *S. marcescens* following knockdown (kd) of each of the *FN3D* genes (Figure 3B). The bacterial load in mosquito guts was determined 5 days post infection by quantitative RT-PCR (qRT-PCR), using both broad range bacterial 16S and *Serratia*-specific primers. Highly significant and robust increase of the *S. marcescens* load was observed after silencing any of the three genes compared to *dsLacZ*-treated controls: 21 to 53-fold in *FN3D1* (Figure 3A), 41 to 60-fold in *FN3D2* (Figure 3B) and 13 to 29-fold in *FN3D3* kd (Figure 3C).

We also assessed the role of *FN3Ds* in shaping the load of *Serratia* naturally found in the mosquito gut. Mosquitoes reared in

![Figure 3. Silencing of *FN3D1-3* increases *Serratia* levels in orally infected mosquitoes or mosquitoes retaining their natural gut microbiota.](image-url)
standard conditions, without antibiotic treatment or infection with *S. marcescens*, were treated with dsRNA against each of FN3D1–3 and the level of commensal *Serratia* was determined 5 days later (Figure 3 A–C, last bar in each panel). Silencing any of the three genes resulted in a significant 4 to 8-fold increase in the levels of commensal *Serratia* compared to dsLacZ-treated controls. These data indicate the involvement of FN3D1–3 in constitutive antibacterial effects that shape the load and composition of the mosquito natural gut microbiota.

**FN3D1–3 kd alters the gut microbiota composition in favor of Enterobacteriaceae**

When the effect of FN3D1–3 kd was assessed on the total bacterial load in the gut of mosquitoes that retained their natural gut microbiota, a non-uniform effect was observed between 4 independent replicate assays (Figure S3). In some cases, FN3D silencing resulted in moderate increases of both *Serratia* and total bacterial load, while in other cases the total bacterial load showed no or marginal increase while *Serratia* showed a strong increase. This variability suggested that the FN3D effect on total bacteria may depend on the initial *Serratia* load and that FN3Ds may function in shaping the population structure of the gut microbiota by affecting a subset of bacteria inhabiting the mosquito gut, including *Serratia*.

To further investigate these hypotheses, we carried out a microbiome analysis using 454 pyrosequencing of samples from two of the replicate assays in which FN3D1–3 kd increased *Serratia* but not total bacteria abundance (Figure 4A-B) and from a replicate assay in which FN3D3 kd increased both *Serratia* and total bacterial load (Figure 4C). The resulting sequence reads were assigned to their respective bacterial family. Reads aligning to *Serratia* reference sequences were categorized separately from other *Enterobacteriaceae* (Table S6).

Considerable variation in bacterial composition was observed in control gut pools between the three assays (Figure 4). This variation is consistent with previously reported metagenomic analyses in lab-reared and field-collected mosquitoes, which revealed extensive gut microbiota diversity at both the individual and population levels [12–14]. Total *Enterobacteriaceae* (*Serratia* and other *Enterobacteriaceae*) were highly prevalent in all pools corresponding to 83.2%, 44.2% and 47.5% of total reads, respectively, while significant variation was observed in the specific representation of *Serratia* that corresponded to 1.9%, 24.5% and 9.5% of total sequence reads, respectively. This natural *Serratia* variation is consistent with the variation observed following oral infection with Db11-GFP *S. marcescens* (see Figure 1) and may be related to the underlying genetic variation. *Acetobacteriaceae* was a prominent family in all assays, while *Flavobacteriaceae* was the prevailing family in the second assay.

In the first assay, FN3D1 or FN3D2 kd increased the representation of total *Enterobacteriaceae* to 87.6% and 89.6%, respectively (Figure 4A). Remarkably, silencing FN3D1 or FN3D2 resulted in a dramatic increase in *Serratia* representation from 1.9% in the dsLacZ-treated control to 30% and 39.3% of total sequence reads, respectively, in agreement with the qRT-PCR analysis of the same samples (Figure S3). Similar results were obtained in the second assay whereby silencing FN3D2 or FN3D3 resulted in an increase in total *Enterobacteriaceae* representation, from 44.2% to 83.1% and 69.7%, respectively (Figure 4B). In both cases, *Serratia* representation showed a precipitous increase from an initial intermediate level of 24.5%, to almost all *Enterobacteriaceae* sequence reads aligning to *Serratia* reference sequences, again in consistence with the qRT-PCR analysis (Figure S3). Although non-*Enterobacteriaceae* representation decreased in both FN3D2 and FN3D3 kd, *Flavobacteriaceae* persisted following FN3D2 kd but were completely eliminated following FN3D3 kd, indicating a difference in the effect between the two FN3Ds related to non-*Enterobacteriaceae* strains.

Taken together, these data indicate that FN3Ds indeed play a major role in shaping the population structure of the mosquito gut microbiota, as silencing any of FN3D1–3 led to increased *Serratia* abundance but also shifted the composition of the mosquito gut microbiota in favor of *Enterobacteriaceae*, mainly *Serratia* or strains that show similarity to *Serratia* reference sequences. This shift may be a result of a specific FN3D function against *Serratia* or a subset of gut bacteria. Alternatively, bacterial interactions or differential growth potential of different bacterial strains may account for the observed shift following a uniform FN3D antibacterial effect.

We tested this hypothesis by examining whether FN3D1–3 silencing could affect the levels of gut infection with non-*Enterobacteriaceae*. Antibiotic treated dsLacZ treated controls and FN3D1–3 kd *Ae. gambiae* mosquitoes were orally infected with bacteria of the genus *Asaia*, a member of the *Acetobacteriaceae* family, common in both field and laboratory-reared *An. gambiae* [9–11] and present in all of our sequenced samples. FN3D1–3 silencing resulted in moderate, non-significant increases in bacterial load, compared to controls (Figure S4), distinguishably lower than following oral *S. marcescens* infection (Figure 3). These data suggest that the observed FN3D antibacterial effect is not uniform across all Gram-negative bacteria and may be specific to a subset of the gut bacterial population including *Enterobacteriaceae*.

The observed shift in favor of *Enterobacteriaceae* representation when both *Serratia* and total bacterial abundance increased following FN3D3 kd was also confirmed by microbiome sequencing that showed an increase of *Serratia* from 9.5% to 33.7% of total sequence reads and of total *Enterobacteriaceae* from 47.5% to 66% (Figure 4C). Remarkably, FN3D3 kd also increased the representation of bacteria of the genus *Burkholderia*, from an initial 0.71% to 15.1% of total reads (Figure 4C). *Burkholderia* were not traced in the dsLacZ treated control pool in which the effect of FN3D3 kd was also assayed (Figure 4B). These data suggest that FN3D3 limits a subset of the mosquito gut bacterial community including *Enterobacteriaceae* but also bacteria of the genus *Burkholderia*.

**Gr9 modulates *S. marcescens* infection levels**

The genomic area encompassing genes encoding the gustatory receptors Gr9 and Gr10 was associated with the outcome of *S. marcescens* infection. As alternative splicing of *Gr9* has been previously suggested [82], with *Gr9* possessing 13 splice variants compared to one for the adjacent *Gr10*, we considered *Gr9* genetic variation more likely to influence the outcome of *S. marcescens* infection, leading to the observed SNP divergence. *Gr9* has shown significant upregulation compared to other tissues in the midgut of blood-fed adult mosquitoes [110] and also in the midgut of adult mosquito tissues [111]. The *Gr9* midgut expression was also confirmed here (Figure S2). Furthermore, comparison of transcription profiles between antennae or maxillary palps and whole body transcriptomes in female mosquitoes has previously shown a non-significant upregulation of *Gr9* in those two tissues (1.42 for antennae and 1.15 for maxillary palps) [112].

We carried out RNAi-mediated silencing of *Gr9* in adult mosquitoes and examined the outcome of oral *S. marcescens* Db11-GFP infection. *Gr9* knockdown resulted in a precipitous 36 to 40-fold increase in *S. marcescens* levels compared to dsLacZ treated controls, as determined using both broad range 16S and *Serratia*-specific primers (Figure 5A). These data suggest that *Gr9* exerts an antibacterial effect that influences the outcome of *S. marcescens* infection.
Figure 4. *FN3D1–3* silencing changes the composition of the mosquito gut microbiota in favor of *Enterobacteriaceae*. The 16S V4–V6 hypervariable regions of gut bacterial populations from mosquitoes retaining their natural gut microbiota without antibiotic treatment or *S. marcescens* infection (*Ab−Sm−*, Figure S3) were sequenced using 454 pyrosequencing (Table S6). cDNA pools from guts of *FN3D1–3* dsRNA treated mosquitoes or *dsLacZ* treated controls, surface sterilized and dissected 5 days post dsRNA treatment, were PCR amplified and sequenced over 3 independent assays (panels A to C). The gut microbiota composition of the *FN3D1–3* dsRNA treated pools or the *dsLacZ*-treated control in each independent assay can be seen in the respective pie charts, with the dsRNA treatment indicated below each pie chart. The color legend indicates the bacterial family corresponding to each pie chart color. Modulation of total bacteria or *Serratia* abundance can be seen for each sequenced pool in Figure S3, with *FN3D1* kd corresponding to replicate 1 in panel 4A, *FN3D2* kd corresponding to replicate 1 in panel 4A and 3 in panel 4B and *FN3D3* kd corresponding to replicate 2 in panel 4B and 4 in panel 4C.

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Transcriptional responses following *S. marcescens* infection

To examine the relationship between genes identified in the population genetics analysis to be associated with the *S. marcescens* infection outcome and infection-induced transcriptional responses, we used DNA microarrays to monitor the transcriptional profile of mosquito guts 3 days post infection with *S. marcescens* added to the sugar meal. Uninfected mosquitoes, which were also treated with antibiotics, were used as controls. Three independent replicate infections were performed. Overall, 55 and 44 transcripts were found to be up and down regulated by at least 1.75-fold, respectively, with 38 and 28 respective up or down regulated transcripts yielding a significant p-value in a t-test against zero, where zero corresponds to no transcriptional regulation (Figure 6A and Table S7). Functional classification of all 97 differentially regulated genes, accounting for multiple transcripts of the same gene, identified serine-type endopeptidases and protein/receptor binding as the most represented classes (Figure 6B). The protein/receptor binding functional class comprised 12 members, including several up or downregulated FREPs, zinc finger containing proteins, PGPRPLC and the complement factor regulator LRIM1, which has been previously shown to be regulated by the IMD/REI2 pathway [113]. The oxidoreductase class comprised 7 members, including two P450 cytochromes, possibly involved in detoxification [114], the hydrolase class included a glycosidase hydrolase and the nucleotide metabolic process class included 5 heat shock proteins, likely to be involved in stress responses [115]. The antimicrobial peptide L15SC2, showing the highest 3.44-fold upregulation of all genes, has been previously shown to be upregulated following a bacterial challenge [116].

A hypergeometric test followed by Benjamini-Hochberg correction was used to determine enriched GO terms in the set of 97 genes. The results identified 16 GO terms that were significantly overrepresented, most of which were related to just two functional classes: serine-type endopeptidases and chitin-binding genes (Figure S6 and Table S8). In total, 16 serine-type endopeptidase genes were differentially regulated, including CLIP6e, which was also associated with the outcome of infection, CLIPB14 that has been implicated in defense against Gram-negative bacteria [57,117], CLIPB17 and CLIPB20. The group of chitin-binding genes comprised 5 members, including the gene encoding the scavenger receptor SCRASP1, previously shown to be upregulated following bacterial infection and bind chitin [117,118] and two downregulated peritrophic matrix components identified by a previous proteomic analysis [119]. Chitin-binding genes are upregulated following oral bacterial infection in *Drosophila* [26], with one member participating in barrier formation that protects against oral *S. marcescens* infection [120], while their suggested role in mosquitoes is recognition of danger signals following tissue remodeling due to a bacterial infection [118].

Several transcriptionally regulated genes suggested a mosquito behavioral response following *S. marcescens* infection (Table 2). Among these genes, NPF was downregulated after infection. NPF is expressed in the midgut of *Drosophila* [121] and *Aedes aegypti* [122], and has been implicated in modulation of feeding behavior in *Drosophila* [30], aversion to noxious food [123] as well as in alcohol sensitivity [80] and regulation of reward systems [81]. It has been also linked to food signaling by integrating sugar gustatory stimuli [124] and behavioral immune responses against endoparasitoid wasps, by mediating oviposition behavior [125].

Additional behavior-related genes that were transcriptionally regulated following *S. marcescens* infection included the gustatory receptor G13 with two downregulated transcripts, three upregulated...
juvenile hormone-inducible kinases, two downregulated genes encoding a pheromone and a juvenile hormone binding protein and the downregulated odorant binding protein genes OBP13 and OBP54. Juvenile hormone circuits are known to affect gustatory perception and feeding behavior in various organisms including Ae. aegypti [126–129], while pheromone and olfaction circuits are also

Figure 6. Transcriptional regulation following S. marcescens infection using DNA microarrays. Antibiotic treated mosquitoes were orally infected with S. marcescens and, 3 days post infection, transcriptional regulation in the gut of bacteria-fed mosquitoes was determined using DNA microarrays, compared to uninfected mosquitoes further antibiotic treated for 3 days. 6A: Volcano plot of transcriptional regulation as determined over 3 independent infections. The log2-transformed fold-change values for each transcript, as determined by two probes for each of the three arrays, were used for a one-sample t-test against zero, where zero corresponds to no regulation. Transcripts with more than 1.75-fold regulation are indicated either by black dots if the p-value of the t-test is >0.05 or red dots if the p-value is <0.05. Transcripts corresponding to LYSC2, PGRPLC, CLIPE6, CLIPE14 and NPF are indicated by arrows. 6B: Functional classification of more than 1.75-fold regulated genes. The 97 genes with more than 1.75-fold regulation were assigned to a functional class based on assigned GO terms, InterPro-predicted domains or Drosophila orthologs. The pie chart shows the proportion of genes assigned to each functional class. Functional classes corresponding to significantly overrepresented GO terms are indicated by asterisks.

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Gr9 modulates *S. marcescens* infection via NPF

We examined a possible link between the observed downregulation of the known modulator of feeding behavior, NPF, following *S. marcescens* infection and the role of Gr9 in modulating the *S. marcescens* infection outcome. The Gr9 ortholog Gr9a has been previously shown to be expressed in the *Drosophila* midgut and co-localize with NPF in enteroendocrine cells [134], raising the possibility of a functional link between these two genes. In mosquitoes orally infected with *S. marcescens*, NPF expression showed a significant 1.9-fold increase following Gr9 silencing compared to dsLacZ treated controls (Figure 7A). This modulation of NPF expression following Gr9 silencing suggested that NPF expression may mediate the observed Gr9 antibacterial effect. Therefore, we further examined whether silencing NPF can affect the increase of *S. marcescens* observed in Gr9 kd mosquitoes. Indeed, concomitant silencing of Gr9 and NPF resulted in a significant 10-fold decrease in infection load, compared to Gr9 silencing alone (Figure 7B).

Taken together, our data suggest a behavioral immune response involving Gr9, mostly relying on changes of NPF expression. One hypothesis is that Gr9 activation in the midgut, most likely through a mosquito-induced cue, tapers the expression of NPF, resulting in feeding suppression that limits the mosquito meal size and thus the abundance of ingested *S. marcescens*. Gr9 variants that influence the efficiency of this suppression may lead to enhanced feeding which, depending on the efficiency of the epithelial response to handle the infection, can influence the outcome of *S. marcescens* infection, thus explaining the observed Gr9 association with the *S. marcescens* infection phenotype.

**Discussion**

The rapidly evolving and adapting mosquito species have become tractable systems for genetic association studies that could...
yield important information about vector/parasite interactions leading to malaria transmission [135]. Previous studies have focused on the outcome of Plasmodium infections, using laboratory or field mosquitoes and genetic tools such as microsatellite markers and targeted SNP loci genotyping [1,3,136]. These studies have not considered the effect of gut bacteria on the outcome of Plasmodium infections, which has been revealed recently [5,16–18]. Furthermore, the influence of associated complement factors on natural P. falciparum infections remains questionable [137]. Indeed, the presence of Enterobacteriaceae, such as S. marcescens, a common member of the mosquito gut flora, has been correlated with P. falciparum susceptibility in field mosquito populations [12], while intraspecific variation within S. marcescens populations also is shown to affect the Plasmodium infection load [37]. Therefore, genome-wide studies to determine factors that modulate the levels of mosquito gut bacteria can provide novel insights into how midgut bacteria affect the outcome of Plasmodium infection and hence malaria transmission.

The unprecedented level of detail achieved in the population genetics analysis presented here in identifying SNPs associated with the outcome of S. marcescens infection is a result of the strong evolutionary drive exerted by gut bacteria on mosquito genetic variation, the use of a high-resolution SNP genotyping array and the use of a recently established laboratory colony of An. gambiae which retains genetic variation found in field populations but also shows elevated linkage disequilibrium due to colonization bottlenecks. This population homogeneity can facilitate gene discovery as shown in human genome-wide association studies in isolated populations [138,139].

A dual implication can be inferred for genes associated with the S. marcescens infection phenotype; they are putatively involved in shaping the infection outcome, while their level of involvement may also be affected by genetic variation within the mosquito population. It is possible that identified associations are the result of causal polymorphisms such as gain or loss of function mutations in coding or regulatory sequences or the result of allele combination in several genetic loci which shapes the outcome of infection through synergism, epistatic interactions or redundant function. In any of the latter cases, a reverse genetics approach may not be capable of capturing such interactions.

The involvement of the three FN3D isoforms in the outcome of Serratia infection reveals a novel function of this family in modulating the load and composition of the mosquito gut microbiota and opens new avenues in investigating the complexity of such responses and possible synergisms with known antibacterial pathways such as the IMD/RELI pathway. The three FN3D genes identified here emerge as major modulators of the bacterial population structure in the mosquito gut, limiting the representation of Enterobacteriaceae, mainly Serratia or strains with similarity to Serratia reference sequences, but also, for FN3D3, bacteria of the genus Burkholderia. As shifts in gut microbiota population structure can elicit gut pathology [28,140], while Serratia can influence the outcome of Plasmodium infection [37], FN3Ds can play critical roles in gut homeostatic interactions and malaria transmission dynamics.

Further insights into the FN3D mode of action remain to be determined. Our data showing that the knockdown effects of FN3D may be limited to Serratia or to a fraction of the microbiota raise intriguing questions about the specificity of bacterial recognition in the mosquito gut. The homology of FN3D2 with the hypervariable pattern recognition receptor Dscam opens the possibility that the specific pathogen recognition shown for AgDscam [47] concerns a broader family of FN3Ds, equipping mosquitoes with the capacity for specific recognition resembling that of animals possessing adaptive immune systems. The phylogenetically unrelated FN3D2 and FN3D3 share a similar domain architecture comprising immunoglobulin and FN3 domains, as is the case with Dscam. The identification of FN3D2 and FN3D3 as being both associated with the outcome of S. marcescens infection and exhibiting discrete but similar phenotypic characteristics in modulating the bacterial population structure in the mosquito gut, parallels the discrete but similar functions of the phylogenetically unrelated Dscam, Frazzled and Roundabout in Drosophila axon guidance, with all three receptors sharing immunoglobulin and FN3 domains [141–144]. FN3D1 has a distinct domain architecture with an FN3 domain, while its orthologous relationship with Drosophila vindes [145] and sequence similarity with the activating transcription factor 7- interacting protein [146], suggest a role in regulating gene expression.

The identification of An. gambiae genes involved in immune responses against bacteria and/or Plasmodium has been largely based to date on studies that combine bioinformatic identification of known immunity gene homologs and transcriptional profiling of genes following a pathogen challenge. This approach, however, has the limitation of the a priori assumption that genes of interest show significant change in transcriptional regulation, mostly induction, which is true for most effectors, but not all genes, for example pattern recognition receptors or transcription factors. In addition, it is possible that even strong changes in transcriptional regulation are the consequence of the infection rather than part of the response. Especially for quantitative traits within mosquito populations, such as Plasmodium infection intensity, different infection intensities can correlate with variable transcriptional responses [70], while the underlying genetic variation further complicates the observed transcriptional regulation.

The microarray approach adopted here has identified a limited set of 99 differentially regulated transcripts following oral S. marcescens infection. The number of regulated transcripts is consistent with that of a previous microarray-based comparison of antibiotic treated and untreated mosquitoes, which showed differential expression for 185 transcripts [16], attributing this limited transcriptional regulation to symbiotic relationships that have led to adaptation of commensal bacteria. A much broader set of differentially expressed genes has been identified following oral bacterial infections in Drosophila [26,32]. This is most likely due to differences in gene pool diversity between the genetically homogeneous fly lines and the recently established mosquito laboratory colony used here, which retains considerable genetic variation thus enabling the SNP genotyping analysis. The different levels of infection seen between mosquitoes (high, low and no infection), which are largely attributed to genetic variation within the colony population, are most likely linked to differences in the mosquito transcription profiles that are averaged out in our study design. Therefore, our analysis identifies transcripts with the most pronounced and consistent differential expression, comprising the core response to S. marcescens infection. Future studies investigating the transcription profile of highly, lowly or non-infected mosquitoes are most likely to reveal components of transcriptional regulation that lead to the respective outcome of infection. Indeed, genes identified to show prominent differential expression after bacterial challenge in previous studies also showed transcriptional regulation following oral S. marcescens infection, including CLIPB14 [117], LRIM1 [63,113], LISC2 [116] and SCRASP [117,118].

The identification of diverse transcriptional responses to different bacteria in Drosophila [32] along with the specificity of mosquito responses to a subset of bacteria, as suggested by the SNP genotyping analysis presented here, may explain the surprisingly little overlap between differentially expressed genes following S. marcescens infection and antibiotic treated vs. untreated
mosquitoes [16]. Remarkably, however, consistency is seen in gene families present in both datasets, including CLIPs, chitin-binding genes, homeobox genes, PGRPs and FREPs, suggesting that similar defense strategies are employed, which are customized for each type of infection through utilization of different gene family members.

The approach we adopted here to identify genes involved in mosquito gut infection with *S. marcescens* combines transcriptional profiling of infected guts with the identification of SNPs segregating between phenotypic pools, whereby an association implies contribution to the outcome of infection, while the study design incorporates variation that leads to different observed phenotypes. This approach addresses some of the aforementioned shortcomings but introduces others, as it cannot capture genes with redundant functions, genes with additional housekeeping functions or a role during development, of which variants are eliminated from the population, or genes with rare variants that are not in the variation pool of our colony. Furthermore, an association may be the result of a selective sweep in the proximity of the gene that creates linkage disequilibrium and leads to SNP divergence between the phenotypic pools. Therefore, although each of the approaches cannot provide by itself a complete picture, the combination of the two can provide novel insights into the mosquito gut responses to *S. marcescens*.

The comparison between the datasets of transcriptionally regulated genes and genes associated with the outcome of *S. marcescens* infection shows limited overlap, with only PGRPLC and CLIPE6 found in both datasets. Again, considerable overlap is detected in identified gene families, which are represented by different members in each dataset. These include acyl-transferase, glycose hydrolyase, kinase, GPCR, LRIM, homeobox, zinc-finger, PGRP, peptidase, FREP, MD2-like and chitin-binding genes. Interestingly, a previous study investigating differential expression following a bacterial challenge in mosquito immunoglobulin-containing genes failed to identify significant regulation for *FN3D2* or *FN3D3* [147], strengthening the case for the complementarity of the SNP genotyping and expression analysis approaches. The specific role of gene family members, especially those showing considerable expansion in *Anopheles*, e.g. FREPs [68, 148], remains unclear. Therefore, SNP genotyping reveals a different set of candidate genes involved in antibacterial immunity while at the same time it is intriguing to postulate whether this divergence between associated and differentially expressed genes within each gene family constitutes a functional divergence between them.

A novel finding stemming from this combinatorial approach is a mosquito behavioral response to *S. marcescens* infection that involves Gr9 signaling and is mediated by changes of NPF expression. Although Gr9 orthologs in *Drosophila* recognize chemosensory cues and mediate aversive behaviors [83, 84, 87], surprisingly, Gr9 appears to suppress feeding irrespective of the presence of bacteria. One explanation is that the Gr9 antibacterial effect relies on its expression in the midgut rather than external sensory organs, where the role of its *Drosophila* counterparts has been studied. The role of gustatory receptor midgut expression [134] remains poorly understood and could involve detection of nutrients or host-derived molecules that triggers downstream responses. The role of NPF midgut expression [121, 122] also remains poorly understood. NPF downregulation following *S. marcescens* infection implies its involvement in an aversion circuit triggered by the presence of *S. marcescens*, with a possible NPF role in integrating aversion and satiation signals that lead to feeding suppression. Such NPF involvement remains to be further investigated, in conjunction with the involvement of other genes related to mosquito behavior which were either associated with the outcome of *S. marcescens* infection or were transcriptionally regulated following infection. These include Gr13, downregulated following *S. marcescens* infection but also three neurotransmitter-triggered GPCRs, associated with the outcome of infection, pointing to complex behavioral circuits involved in antibacterial responses, which are yet to be revealed.

The identification of *FN3Ds* as well as Gr9 and NPF in responses affecting the outcome of *S. marcescens* infection, in addition to known responses including the IMD/REL2 and DUOX pathways, suggests that the response to gut infection is the result of a complex molecular interplay. Both the SNP genotyping and expression analysis suggest that the mosquito response to oral *S. marcescens* infection involves two discrete but inextricably linked modes of defense, a behavioral and an epithelial immune response. A behavioral immune response involving Gr9 and NPF can limit or disrupt pathogen intake, a defense conceptually similar to barrier responses that inhibit pathogen contact with triggers of epithelial or systemic immune responses. An impaired behavioral response, e.g. due to Gr9 variants that affect feeding behavior, can decisively influence the efficacy of the epithelial response and thus the infection outcome. This implies a threshold after which epithelial immunity cannot efficiently handle the pathogen load, an aspect of immunity that remains poorly understood. Nevertheless, in mosquito infections with *Plasmodium* parasites, the intensity of infection has been correlated with the efficacy of different components of the IMD/REL2 pathway, suggesting that different effectors may be deployed in low, mid or high intensity parasite infections [4].

As pathogen abundance most likely relies on feeding behavior, the interplay between behavioral and epithelial immunity can shape both responses. Our implementation of a model of natural bacterial infections through the oral route integrated both behavioral and epithelial responses and not only revealed the previously unknown behavioral component but also allowed the study of aspects of epithelial immunity that, by being infection intensity dependent, possibly rely on the behavioral component. This integrative approach to behavioral and epithelial immunity can be further employed to reveal aspects of this interplay that may involve regulation of behavioral responses by host-derived factors induced by the epithelial component. This implies that the study of behavioral immunity alone may be insufficient to uncover some aspects of its biological consequences.

In *Drosophila*, a balance between immune response and tolerance, achieved by various Imd regulators, largely shapes the gut microbiota population structure, although the only known elicitor of such responses is DAP-type peptidoglycan, common to all Gram-negative bacteria [28, 149, 150]. A similar mechanism has been suggested for mosquitoes through alternative splicing of the modular IMD/REL2 pathway receptor *PGRPLC* that leads to production of positive and negative pathway modulators [3, 42]. Indeed, utilization of alternative splicing as a mechanism to derive new immune functions and increase the specificity of pathogen recognition by the mosquito innate immune system has been described for the *FN3D2* homolog, *Dscam* [43, 44, 47]. Whether the *Enterobacteriaceae*-specific effect of *FN3D2* knockdown is due to specific recognition and activation of highly specialized or targeted effector reactions remains to be investigated. Furthermore, the significance of alternative splicing suggested for Gr9 [82] remains to be determined along with the cue that triggers its antibacterial effect, and could also involve recognition of, most likely, host-derived signals. In addition, recognition of differentially produced metabolites after infection as shown for the DUOX pathway [29] could further increase the specificity in antibacterial responses.
Whether PAMPs (bacterial-derived) or DAMPS (host-derived), such metabolites can be recognized by gustatory receptors triggering specific antibacterial responses, which together with FN2Ds and the rather generalist response of the IMD/REL2 pathway can shape the load and composition of the mosquito gut microbiota. In conclusion, our findings suggest that mosquitoes can mount a much more complex and specific antibacterial response than previously thought, which not only contributes to fending off intestinal bacterial infections but also to achieving homeostasis of the complex gut ecosystem.

Materials and Methods

Ethics statement
This study was carried out in strict accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. The protocols for maintenance of mosquitoes by blood feeding were approved and carried out under the UK Home Office License PPL70/7170. The procedures are of mild to moderate severity and the numbers of animals used are minimized by incorporation of the most economical protocols. Opportunities for reduction, refinement and replacement of animal experiments are constantly monitored and new protocols are implemented following approval by the Imperial College Ethical Review Committee.

Mosquito rearing and maintenance
The N’gousso strain of An. gambiae was used. This is an M form strain colonized in 2006 [1] and kept in large numbers to retain genetic variation. Rearing and maintenance of the strain was performed as described previously [151]. Mosquitoes were collected after emergence and kept on a cocktail of 25 µg/ml gentamicin, 10 µg/ml penicillin and 10 units/ml streptomycin, diluted in 10% D-(-)-Fructose (Sigma). This antibiotic treatment regime was used for 5 days, with the antibiotic solution refreshed every 24 hours. At day 5 post emergence, the antibiotic solution was replaced by dH2O and mosquitoes were starved overnight prior to oral bacterial infection.

Mosquito oral infection with S. marcescens or Asaia
We used the S. marcescens Db11-GFP strain, modified to be GFP-fluorescent and resistant to tetracycline and carbenicillin [152]. S. marcescens glycerol stock was grown in 5 ml LB cultures containing 50 µg/ml tetracycline and carbenicillin (Sigma) at 37°C. Following overnight incubation, the cultures were expanded to 100 ml and further incubated overnight at 37°C. OD600 and GFP fluorescence (excitation/emission at 485/520 nm) were then measured to ensure cultures maintained GFP fluorescence, using the Fluostar Omega spectrophotometer (BMG Labtech). Bacterial pellets following centrifugation at 2500 rpm for 5 minutes were washed twice with PBS and resuspended in such volume of 10% D-(-)-Fructose, so that 1 ml of the bacteria-containing sugar solution corresponded to OD600 = 0.1 of the initial 100 ml culture. The sugar solution was further diluted 1:12 in a 10% D-(-)-Fructose solution that contained tetracycline and carbenicillin at 50 µg/ml and 5% v/v of scarlet dye (Langdale). Mosquitoes were fed with this solution for 2 days. Subsequently, mosquitoes fed with bacteria-containing sugar were separated based on the presence of the dye in their gut and kept on 10% D-(-)-Fructose containing tetracycline and carbenicillin at 50 µg/ml.

Oral infections with Asaia were conducted in a similar manner. The Asaia SF2.1 (GFP) strain was used, grown as previously described [153] and maintained in 50 µg/ml kanamycin (Sigma).

Fluorescence microscopy
The levels of S. marcescens infection were determined by microscopic observation of dissected midguts immersed in Vectashield mounting medium (Vector), immediately after dissection. The Zeiss Axioshot fluorescence microscope was used, equipped with light and GFP filters while photos of observed midguts were taken with the AxioCAM HRc and Axiovision software (Zeiss).

SNP genotyping arrays
All carcasses arrays to midguts of S. marcescens infected mosquitoes were kept numbered in 96-well plates immersed in 75% ethanol at −80°C. Carcasses from selected midguts were used for gDNA extraction using the QiAquick Blood and Tissue kit (Qiagen). Subsequently, gDNA concentrations were determined using the PicoGreen dsDNA kit (Invitrogen) and equimolar gDNA quantities from each mosquito were pooled. The design and validation of the SNP genotyping array used along with the treatment of gDNA pools, hybridization, calling of SNP genotypes and measurement of differentiation in each pooled hybridization between allele A and B have been described previously [39,154]. The frequency of designated allele A was considered as the minor allele frequency and was used to measure the difference between pooled hybridizations. The permutation analysis used has been described previously [39], with a modified length of non-overlapping 10-SNP windows. Determination of genes residing in identified genomic areas and homology analysis was performed using BioMart 0.7 and the AgamP3.7 An. gambiae gene annotation [155]. The SNP genotyping array datasets have been deposited to ArrayExpress under the experiment name Serratia_SNPI and accession number E-MEXP-3951.

DNA microarrays
Total RNA was extracted from midguts using the Trizol reagent (Invitrogen), and treated with Turbo DNAse I (Ambion). Samples were further purified using the RNeasy kit (Qiagen). Quantification was performed using the Nanodrop 1000 spectrophotometer (Thermo Scientific) and RNA integrity was assessed using the RNA 6000 Pico Chip kit (Agilent). Labeling and hybridization were performed using the Low Input Quick Amp Labeling kit for two-color microarray based expression analysis (Agilent). We used Agilent custom 4×44 k gene expression microarrays. The microarray design Pfalcip_Agam2009 (A-MEXP-2324) comprises oligonucleotide probes encompassing all An. gambiae annotated transcripts of the AgamP3.6 release along with P. falciparum probes, with each probe represented in duplicate. Slides were scanned using the Genepix 4000B scanner equipped with the Genepix Pro 6.1 software (Axon instruments).

All dataset files were normalized using the GeneSpring 11.0 GX software (Agilent). The Lowess normalization method was used while the threshold of raw signals was set to 5, which was sufficient to eliminate background regulation of P. falciparum probes. Further analysis of transcriptionally regulated genes and GO analysis was performed using the Genespring 11.0 GX software. For GO analysis, GO accession numbers for all An. gambiae transcripts were obtained using Biomart 0.7 and a hypergeometric test with Benjamini-Hochberg correction was performed on the set of more than 1.75-fold regulated genes. The corrected p-value for testing multiple GO accession numbers for their significance was set to 0.1. The log2-transformed transcriptional regulation for each transcript was extracted from the normalized datasets for each of the two probes corresponding to each transcript and the obtained values from all three independent infections were used in a t-test against zero, with a p-value cut-off of 0.05. The DNA microarray datasets
have been deposited to ArrayExpress under the experiment name Serratia_infections and accession number E-MEXP-3952.

Bacterial load following RNAi-mediated silencing
Mosquitoes were treated with the respective dsRNA at the day of emergence, as described previously [156]. For each targeted gene or the dsLacZ control, dsRNA was synthesized using the T7 Megascript kit (Invitrogen) and further purified using the RNeasy kit (Qiagen) to a concentration of 3 μg/μl. For each T7 Megascript reaction, 1 μg of purified PCR product was added, derived using the T7 primer sets shown in Table S9, using An. gambiae cDNA as template.

Mosquitoes were surface sterilized by immersing them in 70% ethanol for 30 seconds and washing them twice in PBS and midguts were dissected in RNA later (Invitrogen). Total RNA from mosquito midguts was extracted after homogenization with a pestle motor in RNA later using the RNeasy kit (Qiagen). cDNA was synthesized from total RNA using the QuantiTect Reverse Transcription kit (Qiagen).

Quantification of bacterial load or the efficiency of RNAi-mediated silencing was performed using qRT-PCR with the respective primers shown in Table S9. In a 20 μl reaction of Fast SYBR Green Master Mix (Applied Biosystems), 1 μl of cDNA template and 2 μl of each respective primer at a 0.5 to 9 μM concentration, optimized for each primer set, were added. The 7500 Real-Time PCR System (Applied Biosystems) was used with its respective software to perform the reaction and any further analysis. The relative abundance of each sample was determined using the standard curve method as described in User Bulletin #2 for the ABI Prism 7700 Sequence Detection System (Applied Biosystems) in which the housekeeping AgS7 gene was used as an endogenous control.

454 pyrosequencing
cDNA pools were amplified with the GO Taq DNA polymerase (Promega) using the 16S V4–V6 primers shown in Table S9 and suitable barcode sequences and purified using PCR purification and Gel Extraction kits (Qiagen). PCR products were sequenced by Beckman Genomics (Grenoble, France) using the Roche 454 GS FLX+ and standard procedures. The resulting FASTA files were filtered to a minimum read length of 250 bp using Galaxy [157] and blasted against the NCBI 16S microbial database using BLAST+ and infernalBLAST [156] with standard blastn algorithm settings and 10 maximum target sequences. Further analysis was performed using MEGAN4 [159].

Meal size and two-choice preference assays
Sugar meal size was determined through a modified capillary feeder assay [160]. Mosquitoes treated with LacZ or Gr9 dsRNA were antibiotic treated for 5 days, starved overnight and, subsequently, individual mosquitoes were fed on a 5 μl glass capillary (VWR) containing 10% D-(-)-Fructose and 5% v/v scarlet dye. For alive mosquitoes, sugar consumption was determined 16 hours later through the reduction of sugar solution in each capillary. The two-choice preference assay was also conducted based on a previously described capillary feeder assay [160]. Mosquitoes treated with LacZ or Gr9 dsRNA were antibiotic treated for 5 days, starved overnight and placed in pools of 8–11 mosquitoes. Mosquitoes were offered to feed from two capillaries, one containing a sugar solution as above and one also containing S. marcescens, prepared as described above for oral infection. Water-containing cotton pads were also used and pools with mosquito mortality were disregarded. 16 hours later, consumption for each capillary was determined based on the reduction of the sugar solution.

Supporting Information
Figure S1 Efficacy of antibiotic treatment in reducing the presence of gut bacteria in treated mosquitoes. An. gambiae mosquitoes were antibiotic treated for 5 days with a cocktail of gentamicin, penicillin and streptomycin. Subsequently, 10–15 mosquitoes were surface sterilized and their guts were dissected, homogenized and total RNA was extracted and further used for cDNA synthesis. Mosquitoes kept untreated were processed in the same way. Subsequently, cDNA from antibiotic treated or untreated mosquito pools was used in a qRT-PCR using broad range bacterial 16S primers while AgS7 primers were used as controls. The bacterial load ±SEM in 3 independent assays, with the qRT-PCR performed at least twice for each assay, can be seen. Asterisks indicate significance, with a p-value<0.0005, in a Mann-Whitney non-parametric test between the bacterial load of untreated and antibiotic treated mosquitoes. (TIF)

Figure S2 Efficacy of RNAi-mediated silencing of FN3D1–3, Gr9 and NPF. Mosquitoes were treated with dsRNA targeting the FN3D1, FN3D2, FN3D3, Gr9 or NPF transcripts or with the dsLacZ control. The Gr9 dsRNA targets the 5’ exon 1, which mainly limits the RA and RG splice variants. The relative expression of each transcript in the mosquito gut was determined 5 to 6 days post dsRNA treatment, normalized to the endogenous AgS7 control, in mosquitoes treated with the respective dsRNA by qRT-PCR and primers targeting the respective transcript in a region not targeted by the respective dsRNA. Silencing efficiency was determined by further normalizing the relative expression of each transcript in mosquitoes treated with the respective dsRNA to the relative expression in the dsLacZ treated control. The average ±SEM of relative expression is shown for at least 3 independent assays, with the qRT-PCR performed at least twice for each assay. (TIF)

Figure S3 FN3D1–3 silencing modulates total bacteria and Serratia in a non-uniform way in mosquitoes retaining their natural gut microbiota. Mosquitoes retaining their natural gut microbiota (Δb−Sm−) were treated with FN3D1 (S3A), FN3D2 (S3B) or FN3D3 (S3C) dsRNA and bacterial load was normalized to the respective dsLacZ treated control. Bacterial load using broad range 16S or Serratia-specific primers is shown for 4 independent assays (Replicates 1–4 as indicated above each pair of bars). (TIF)

Figure S4 Oral infection with Asaia following FN3D1–3 silencing. Antibiotic treated mosquitoes were orally infected with bacteria of the genus Asaia following treatment with FN3D1–3 dsRNA or the dsLacZ control. Bacterial load was determined in the guts of surface sterilized mosquitoes dissected 5 days post infection, using qRT-PCR with 16S broad range bacterial primers and the AgS7 control. The bacterial load ±SEM in 3 independent assays, with the qRT-PCR reaction performed at least twice for each assay, can be seen. (TIF)

Figure S5 Two-choice preference assay between sugar solutions containing or not S. marcescens. Antibiotic treated mosquitoes treated either with LacZ or Gr9 dsRNA were starved overnight and, subsequently, pools of 8–11 mosquitoes were offered a choice of two meals in separate 5 μl capillaries, one containing a sugar solution containing a dye used to measure
consumption and ensure uptake from the mosquitoes and one also containing *S. marcescens*, at the same concentration as used for oral infection. 16 hours later, consumption was measured for each capillary and, for each mosquito pool, the ratio of the % consumption in the *Serratia*-containing capillary to the % consumption in the sugar-only capillary was determined. Overall, the consumption percentage ratio of *Serratia*-containing versus sugar-only capillaries was determined for 6 *LacZ* and 11 *Gr9* dsRNA treated mosquito pools. The average ±SEM percentage ratio for each dsRNA treatment can be seen. Significant differences were assessed using the non-parametric Mann-Whitney test resulting in a p-value of 0.7395, indicated as non-significant (NS). A percentage ratio of >100% would indicate aversion to the *Serratia*-containing solution while a percentage ratio of >100% would indicate attraction to the *Serratia*-containing solution for mosquitoes treated with the respective dsRNA, as indicated. (TIF)

**Figure S6 Significantly overrepresented GO terms in the set of more than 1.75-fold regulated genes following *S. marcescens* infection.** A hypergeometric test with Benjamini-Hochberg correction was used to compare the representation of genes corresponding to the same GO term in the set of 97 more than 1.75-fold regulated genes following *S. marcescens* infection to the respective representation in the *An. gambiae* genome, as annotated in the *Ptilicp_Agamb2009* microarray design. 16 GO terms corresponded to significantly overrepresented groups of genes, shown in orange type in the GO directed acyclic graph, with GO terms in the same path that did not meet the p-value cutoff shown in black type. For significantly overrepresented GO terms, the number of corresponding genes in the set of more than 1.75-fold regulated genes is shown in parenthesis. A table with the regulated transcripts corresponding to GO terms at each final leaf node is shown, including, for each transcript, the Transcript ID, an assigned name or description based on *Interpro*-predicted domains or homologies with *Drosophila* counterparts and the observed transcriptional regulation following *S. marcescens* infection. (TIF)

**Table S1 Complete list of SNP loci assayed for association with the *S. marcescens* infection phenotype.** SNP loci interrogated in a 400 k Affymetrix SNP genotyping array. The MAF for the highly infected and non-infected phenotypic pools and the calculated MAF difference can be seen for each interrogated SNP locus. (RAR)

**Table S2 Complete list of 10-SNP loci windows assayed for association with the *S. marcescens* infection phenotype.** The 40,007 sliding 10-SNP windows assayed for association with the *S. marcescens* infection phenotype are shown along with the genomic position of each 10-SNP window, the calculated average MAF difference and the assigned p-value of the permutation analysis conducted. (XLSX)

**Table S3 Individual SNP loci associated with the *S. marcescens* infection phenotype.** A total of 140 out of 400,071 SNP loci that showed MAF difference >0.5 between the highly infected and non-infected *S. marcescens* phenotypic pools are shown. Each column contains for each SNP locus: the chromosome it resides, the MAF for the highly infected and non-infected phenotypic pools and the calculated MAF difference, the genomic position of the SNP locus and the calculated 5 kb radius (position −5 kb and position +5 kb columns), the residing peak for each SNP locus as assigned in Figure 2 and genes found within a 5 kb radius of this SNP locus based on the AgamP3.7 annotation of the *An. gambiae* genome. (XLSX)

**Table S4 10-SNP loci windows associated with the *S. marcescens* infection phenotype.** A total of 44 out of 40,007 10-SNP loci windows showed a significant p-value of <10⁻⁵ in a permutation analysis following Bonferroni correction, when assayed for association with the *S. marcescens* infection phenotype. Each column contains for each 10-SNP locus window: The chromosome it resides, the genomic position (Center-Start-Stop columns), the average MAF difference calculated, the assigned p-value of the permutation analysis, the peak it resides as assigned in Figure 2 and the genes residing within the 10-SNP locus window based on the AgamP3.7 annotation of the *An. gambiae* genome. (XLSX)

**Table S5 Complete list of *An. gambiae* genes associated with the outcome of *S. marcescens* infection.** 138 *An. gambiae* genes were associated with the outcome of *S. marcescens* infection, either by residing within a 5 kb radius of SNP loci with MAF difference >0.5 (SNP) or by residing within 10-SNP loci windows with a <10⁻⁵ p-value in a permutation analysis (Permutation). For genes identified by both approaches, both SNP and Permutation are noted. For each gene, the Description column includes the assigned name, if any, or a description based on homology or *Interpro*-predicted domains. (XLSX)

**Table S6 16S metagenomic profiling in FN3D1–3 or *LacZ* dsRNA treated mosquitoes retaining their natural gut microbiota.** cDNA pools from *Ab–Sm−* mosquitoes following FN3D1–3 or *LacZ* dsRNA treatment were sequenced using 454 pyrosequencing targeting the V4–V6 region of the bacterial 16S rRNA transcript. Results are shown for each cDNA pool corresponding to a dsRNA treatment shown in Figure 4. In each case, taxonomic identification and alignment of reference sequences for each identified taxon is shown along with the number of sequence reads assigned. For each bacterial genus, the reference sequence with most alignment hits (sequence reads) is shown. For *Serratia*, the reference sequence with most alignment hits is highlighted and was used for categorization between *Serratia* and other *Enterobacteriaceae*. (XLSX)

**Table S7 *An. gambiae* transcripts with more than 1.75-fold regulation following *S. marcescens* infection.** 99 *An. gambiae* transcripts showing more than 1.75-fold up or down regulation following *S. marcescens* infection. For each transcript, the Transcript ID is shown along with its designated name or functional description, the assigned functional class, the fold-change regulation and the p-values obtained by a t-test against zero using the log2-transformed values for each interrogated probe. (XLSX)

**Table S8 GO terms significantly overrepresented in the set of more than 1.75-fold regulated genes following *S. marcescens* infection.** 16 enriched GO terms as determined by a hypergeometric test followed by Benjamini-Hochberg correction using a 0.1 p-value cut-off are shown. The GO ID, accession number and description of each GO term is shown along with the results of the test for each GO term. (XLSX)

**Table S9 Sequences of primers used for qRT-PCR, dsRNA synthesis or 454 pyrosequencing.** For each forward
or reverse primer, its use is indicated along with the respective sequence. (XLSX)

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Author Contributions

Conceived and designed the experiments: GKC SS. Performed the experiments: SS. Analyzed the data: SS. Contributed reagents/materials/analysis tools: GKC MKNI DEN MATM. Wrote the paper: SS GKC.

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