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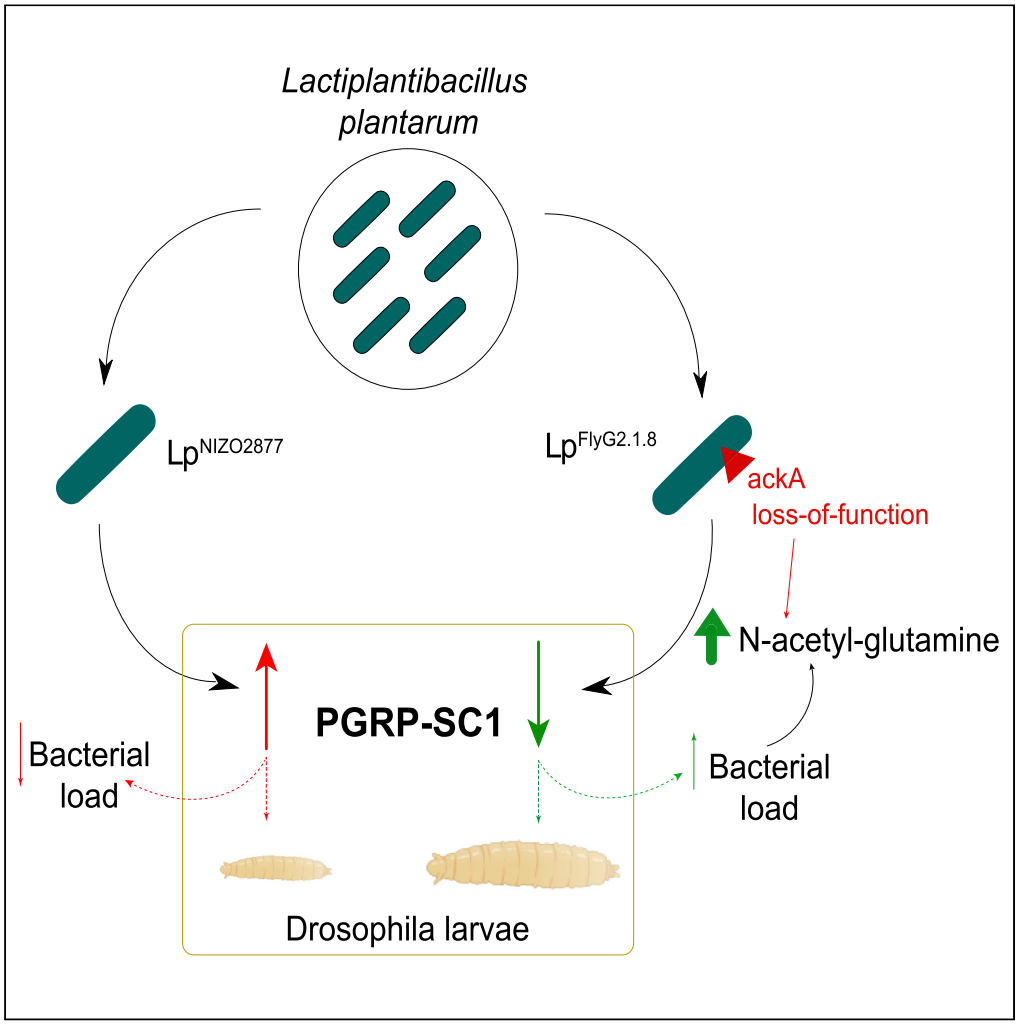

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Highlights

Beneficial *L. plantarum* strains downregulate the expression of *Drosophila* PGRP-SC1

L. plantarum proliferation triggers the lower expression of *Drosophila* PGRP-SC1

Downregulation of PGRP-SC1 relies on the loss of function of *L. plantarum ackA* gene

PGRP-SCs downregulation is sufficient to improve *L. plantarum* beneficial effect

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Beneficial commensal bacteria promote Drosophila growth by downregulating the expression of peptidoglycan recognition proteins

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SUMMARY

Commensal bacteria are known to promote host growth. Such effect partly relies on the capacity of microbes to regulate the host's transcriptional response. However, these evidences mainly come from comparing the transcriptional response caused by commensal bacteria with that of axenic animals, making it difficult to identify the animal genes that are specifically regulated by beneficial microbes. Here, we employ *Drosophila melanogaster* associated with *Lactiplantibacillus plantarum* to understand the host genetic pathways regulated by beneficial bacteria and leading to improved host growth. We show that microbial benefit to the host relies on the downregulation of peptidoglycan-recognition proteins. Specifically, we report that bacterial proliferation triggers the lower expression of PGRP-SC1 in larval midgut, which ultimately leads to improved host growth and development. Our study helps elucidate the mechanisms underlying the beneficial effect exerted by commensal bacteria, defining the role of immune effectors in the relationship between Drosophila and its gut microbes.

INTRODUCTION

Metazoans live in constant association with microbial communities, and their reciprocal interaction is known to favor many traits of both partners' physiology (Fraune and Bosch, 2010; McFall-Ngai et al., 2013). On one hand, animal hosts can benefit the growth and metabolism of their microbial partners, specifically through the secretion of a complex blend of metabolites (Storelli et al., 2018; Wier et al., 2010; Wilson et al., 2010). On the other hand, beneficial bacteria sustain animal health by contributing to the maintenance of the immune system homeostasis (Sommer and Bäckhed, 2013), confer protection against pathogen infections (Blum et al., 2013; Fraune et al., 2015), promote intestinal epithelium renewal (Buchon et al., 2009; Shin et al., 2011), and influence host lifespan and development (Brummel et al., 2004; Guo et al., 2014; Ren et al., 2007). In addition, the gut microbiota bolsters the host digestive processes through the production of a large collection of enzymes that favor the metabolism of dietary molecules as well as specific organic compounds such as vitamins, amino acids, and short-chain fatty acids (Nicholson et al., 2012; Wilson et al., 2010). This type of relationship is referred to as nutritional symbiosis (Hooper et al., 2002). Most insects undertake facultative nutritional symbioses with their microbial communities (Douglas, 2011; Pontes and Dale, 2006), where both partners benefit from each other's presence without relying on one another for their survival (Gilbert and Neufeld, 2014). *Drosophila melanogaster*, associated with its microbiota, is a well-known example of such relationship (Erkosar et al., 2013; Lee and Brey, 2013) and represents a powerful model to study host-microbe interactions (Buchon et al., 2013; Ma et al., 2015). Wild and lab-reared Drosophila are associated with relatively simple beneficial bacterial communities. These communities mainly include *Acetobacteraceae* and *Lactobacillaceae* families, with *Acetobacter pomorum and Lactiplantibacillus plantarum* as the most common species of the *Drosophila melanogaster* microbiota (Chandler et al., 2011; Erkosar et al., 2013; Wong et al., 2011). *Drosophila* microbiota influences the host's physiology throughout its entire life (Buchon et al., 2013; Erkosar et al., 2013; Lee and Brey, 2013). Beside the abovementioned benefits, fly gut microbes are also known to impact their host's physiology via regulation of many host transcriptional pathways (Nichole A. Broderick et al., 2014). Indeed, numerous studies have been conducted to characterize the impact of the microbiota on the gene expression of the adult fly. Commensal bacteria are involved in the modulation of intestinal immune homeostasis (Bosco-Drayon et al., 2012; Bost et al., 2018; Lhocine et al., 2008; Paredes et al., 2011),

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they shape gut morphology through enhancement of stem cell proliferation and epithelial renewal rate (Nichole A. Broderick et al., 2014; Ridley et al., 2012; Shin et al., 2011), and they have also been shown to mediate transgenerational inheritance of responses to environmental changes (Karunakar et al., 2019; Zare et al., 2018). In addition, it has been demonstrated that gut microbes influence host phenotype by promoting the coexpression of group of genes, such as the insulin-like signaling (IIS) and target of rapamycin (TOR) signaling pathways, transcription factors including the IMD factor *Relish*, and many more genes, involved in coordinating cellular signaling, growth, and metabolism (Dobson et al., 2016).

One of the most striking beneficial effects exerted by gut microbes is host growth promotion (Consuegra et al., 2020b; Schwarzer et al., 2016; Storelli et al., 2011). Some bacterial species associated with *D. melanogaster* have been demonstrated to be able to promote fly growth and maturation rate during the juvenile phase in poor-nutrient conditions (Storelli et al., 2011). The growth-promoting ability of the gut microbiota is strain dependent and is exerted through the enhancement of the systemic production of two hormonal growth signals: Ecdysone (Ecd) and Drosophila insulin-like peptide (dILP) via increased TOR kinase activity in both fat body and prothoracic gland (Erkosar et al., 2015; Storelli et al., 2011). In addition, it has been shown that fly growth promotion mediated by gut microbes results, at least in part, from the upregulation of digestive enzyme expression in the midgut via IMD/Relish activity (Erkosar et al., 2015). These insights came from comparing Drosophila with a microbiota (either single-strain associations or complex communities) versus without a microbiota (germ-free condition) (Bost et al., 2018; Nichole A. Broderick et al., 2014; Delbare et al., 2020; Dobson et al., 2016; Erkosar et al., 2015; Paredes et al., 2011), and they largely contributed to our knowledge of the molecular mechanisms underlying the role of gut microbes in host physiology. Nevertheless, not all bacterial species or strains associated with Drosophila microbiota are beneficial. As a consequence, a clear understanding of the host genetic pathways that are specifically regulated by beneficial bacteria remains elusive.

We recently demonstrated that a strain of *L. plantarum* ($Lp^{NIZO2877}$) bearing a moderate *Drosophila*-growth-promoting ability was able to improve its beneficial effect by adapting to the fly nutritional environment (Martino et al., 2018). The $Lp^{NIZO2877}$-evolved strain ($Lp^{Fly.G2.1.8}$) showed a single mutation in the acetate kinase A (*ackA*) gene, causing the increased production of N-acetylated amino acids, which were sufficient to improve *L. plantarum*-growth-promoting capacity. In this study, we coupled comparative transcriptome analyses and CRISPR-Cas-based microbial engineering on such isogenic strains to reveal the specific host transcriptional pathways regulated by the association with beneficial gut bacteria and leading to enhanced animal growth. The presence of a single genetic difference between the bacterial strains, which is responsible for the improvement in growth promotion, allowed us to uniquely ascribe the differential host transcriptional response to the microbial benefit. We observe that growth-promoting *L. plantarum* strains are able to specifically lower the expression of the *Drosophila* peptidoglycan recognition protein (PGRP) SC1, whose activity is regulated by *L. plantarum ackA* gene function and its metabolic products. In addition, we demonstrate that the downregulation of *PGRP-SC1* gene in larvae associated with a mid-beneficial strain is sufficient to recapitulate the beneficial effect of the strong growth-promoting strain and that such benefit is directly linked to bacterial growth.

Our study advances the current knowledge in host-microbe interactions by demonstrating that commensal bacteria improve fly growth by actively regulating the expression of immune effectors; this leads to higher bacterial proliferation and the increased production of beneficial microbial metabolites, which are then consumed by the host. Our results contribute to shed light in the complex mechanisms underlying the relationships between host and their gut microbes.

RESULTS

Commensal bacteria significantly affect *Drosophila* transcriptional response

To unravel the *Drosophila* transcriptional pathways specifically regulated by beneficial gut microbes, we compared the transcriptome of *yw D. melanogaster* larvae during the second instar associated with two strains of *L. plantarum* promoting Drosophila growth to different extents ($Lp^{NIZO2877}$ *and* $Lp^{FlyG2.1.8}$, gnotobiotic larvae) with germ-free (GF) larvae as negative control. $Lp^{NIZO2877}$ is a *L. plantarum* strain isolated from processed human food (Martino et al., 2015a), showing a moderate growth promotion both in *Drosophila* and in mice (Schwarzer et al., 2016). $Lp^{FlyG2.1.8}$ is a $Lp^{NIZO2877}$-derived strain, experimentally evolved in poor-nutrient conditions in the presence of *D. melanogaster*. $Lp^{FlyG2.1.8}$ is isogenic to $Lp^{NIZO2877}$, with one exception: $Lp^{FlyG2.1.8}$ bears a tri-nucleotide deletion in the *ackA* gene, which is responsible for a

significant improvement in larval growth promotion compared with its ancestor (Martino et al., 2018). The rationale behind the choice of such strains is that any differences in host transcript levels could be directly ascribed to the single bacterial genetic mutation and, thus, to the improved growth-promoting effect. Following bacterial association with GF embyos, we performed Drosophila RNA extraction on size-matched larvae (2.4 mm length). Specifically, considering that microbiota-associated larvae develop faster than axenic larvae (Storelli et al., 2011), we collected the *Lp*-associated larvae 4 days after monoassociation, whereas the GF larvae were collected after 5 days (Figure S1). The transcriptome sequencing of Drosophila larvae yielded 47–31 million reads for all replicate samples. We analyzed the transcripts using a dedicated R script and obtained a data set of 17,560 genes (Table S1). Among these genes, 559 genes were differentially expressed between axenic and gnotobiotic larvae that satisfied our criteria for significant differential expression (p-value < 0.05 and −1.5- to +1.5-fold). Two hundred eighty-five genes were differentially expressed in both gnotobiotic conditions ($Lp^{NIZO2877}$- and $Lp^{FlyG2.1.8}$-associated larvae) compared with GF larvae, among which 106 resulted to be upregulated and 179 downregulated (Table S1). Next, we analyzed the remaining 274 genes, and we observed that 209 were differentially expressed between $Lp^{FlyG2.1.8}$-associated larvae and GF larvae, with 96 upregulated and 113 downregulated genes, whereas the other 65 genes were differentially expressed between $Lp^{NIZO2877}$-associated larvae and GF larvae, with 22 upregulated and 43 downregulated (Figure S2A, Table S1).

We assigned biological pathways to the identified 559 genes using DAVID Gene Functional Classification Tool (Huang et al., 2009), which annotates each gene and identifies the most relevant biological terms associated with a given gene. To improve the usefulness of the functional annotation analysis, we carried out DAVID clustering analysis for a group of up- and downregulated genes, respectively, and we considered only the genes that met our statistical criteria (Benjamini adjusted p-value < 0.1).

The transcripts enriched in gnotobiotic larvae compared with GF larvae were dominated by the expression of lysozyme genes (i.e., *LysB, C, D* and *E*) involved in the hydrolysis of beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan and the expression of heat shock protein (*Hsp*) genes (i.e., *Hsp22, 23, 26, 27* and *28*) commonly expressed in response to stress resistance and adaptation (Figure S2B, Table S2). Furthermore, gnotobiotic larvae showed significantly higher expression of immunity-related genes, including genes involved in peptidoglycan recognition and the negative regulation of innate immune response, such as *PGRP-SB1*, *PGRP-SD*, *PGRP-LA*, and *PGRP-SCs* (Tables S1 and S2). The genes downregulated in gnotobiotic larvae compared with germ-free conditions were implicated in membrane function through the involvement of genes with catalytic activity, such as *Pkn* and *Adgf-A2* (Tables S1 and S2).

By comparing the transcriptional response between single-strain associated larvae and GF larvae, the GO term analysis revealed an enrichment of expression in genes implicated in DNA methylation (*Mt2*), DNA binding, and DNA-directed RNA polymerase activity (*Rpb5, RpI12*) in $Lp^{FlyG2.1.8}$-associated larvae. In addition, we detected a significant decrease in the expression of genes involved in cell biogenesis, such as *l(2)gl*, the *crb* gene essential for the development of polarized epithelia and centrally involved in cell polarity establishment, and the *scrib* gene required for polarization of the embryonic imaginal disk and follicular epithelia (Figure S2C, Table S2).

Highly beneficial *L. plantarum* strains trigger downregulation of the peptidoglycan-recognition protein SC1 compared with mid-beneficial strains

Next, we sought to identify the host genes regulated by a beneficial bacterium, specifically improving fly growth. To do this, we compared the transcriptome profiles between $Lp^{NIZO2877}$- and $Lp^{FlyG2.1.8}$-associated larvae. Our analysis identified 21 genes differentially expressed between the conditions (p < 0.05 and −1.5- to +1.5-fold), with 6 and 15 genes being down- and upregulated in $Lp^{FlyG2.1.8}$-associated larvae compared with $Lp^{NIZO2877}$-associated larvae, respectively (Figure 1, Table S3). The transcripts found to be enriched in larvae associated with the beneficial strain $Lp^{FlyG2.1.8}$ were dominated by the expression of the Sperm-Leucylaminopeptidase 3 (*S-Lap3*) gene (22.8 fold), a major constituent of paracrystalline material from *D. melanogaster* sperm involved in the fertility of male flies (Laurinyecz et al., 2019). We also observed the upregulation of genes involved in the production of larval cuticle protein (*Lcp4* and *Lcp65Ab1*), proteolysis (*CG43124* and *CG43125*), signal peptides (*CG14258, CG14259*, and *CG15597*), the odorant-binding protein 56e (*Obp56e*) involved in nutrient sensing, and methuselah-like 6 (*mthl6)* implicated in Drosophila development and lifespan. At the same time, $Lp^{FlyG2.1.8}$-associated larvae showed downregulation of

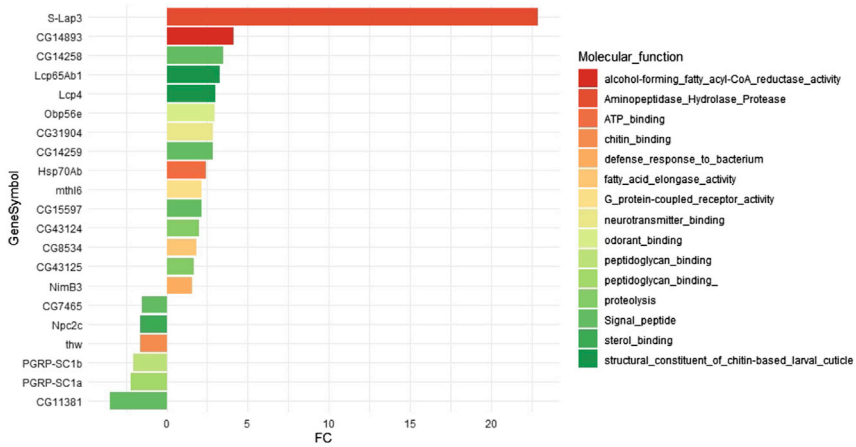

Figure 1. *Lp*$^{FlyG2.1.8}$ significantly alters Drosophila transcriptional response compared with *Lp*^{NIZO2877}

Bar chart of the genes with significantly differential expression (up- and downregulated) between *Lp*$^{FlyG2.1.8}$- and *Lp*^{NIZO2877}-associated larvae ($p < 0.05$ and −1.5- to +1.5-fold). Each color refers to the molecular function associated with each gene.

genes involved in chitin metabolism (*thw*), the Niemann-Pick type C-2c (*Npc2c*) gene, implicated in sterol transport, and two signal peptides (*CG11381, CG7465*) (Table S4). Surprisingly, we found that *Lp*$^{FlyG2.1.8}$-associated larvae showed a lower expression of *PGRP-SC1a* and *PGRP-SC1b* genes compared with *Lp*^{NIZO2877}-associated larvae. No difference was detected in the regulation of other PGRPs (Tables S1 and S4). The specific lower expression of *PGRP-SC1* in larvae associated with the strong growth-promoting strain *Lp*$^{FlyG2.1.8}$ prompted us to investigate the causes of such immune system downregulation.

Drosophila PGRP-SC1 expression relies on *L. plantarum ackA* function

First, to validate the RNA-Seq data, transcript abundance of *PGRP-SC1* gene was assessed on size-matched larvae newly associated with *Lp*^{NIZO2877} and *Lp*$^{FlyG2.1.8}$ strains. The amplification results confirmed a significantly lower expression of *PGRP-SC1* in *Lp*$^{FlyG2.1.8}$-associated larvae (Figure 2A). Drosophila PGRP-SCs are known to be mainly expressed in the larval gut (Bischoff et al., 2006; Costechareyre et al., 2016). To rule out the possibility that the difference of PGRP-SC1 levels was due to variation of expression in different tissues, we tested the expression of PGRP-SC1 on dissected larval guts. We detected lower levels of PGRP-SC1 in the guts of *Lp*$^{FlyG2.1.8}$-associated larvae compared with *Lp*^{NIZO2877}-associated larvae (Figure 2B), reflecting the expression levels obtained by analyzing whole larvae.

As *Lp*$^{FlyG2.1.8}$ has been experimentally co-evolved with the fruit fly, we hypothesized that the downregulation of *PGRP-SC1* expression might be due to mechanisms of immune tolerance. To test this hypothesis, we analyzed the expression of *PGRP-SC1* in the guts of larvae associated with *Lp*$^{DietG20.2.2}$, a different *Lp*^{NIZO2877}-derived strain experimentally evolved on the fly diet and bearing a mutation in the same *ackA* gene as *Lp*$^{FlyG2.1.8}$ that also improved growth promotion (Martino et al., 2018). *Lp*$^{DietG20.2.2}$ triggered the downregulation of *PGRP-SC1* compared with *Lp*^{NIZO2877}-associated larvae (Figure 2B), thus suggesting that *PGRP-SC1* expression was independent of the strain evolutionary history. Next, we focused on the microbial molecular mechanisms that led to the downregulation of *PGRP-SC1* expression. Specifically, both *Lp*$^{FlyG2.1.8}$ and *Lp*$^{DietG20.2.2}$ strains bear nonsynonymous mutations in the *ackA* gene, which have been predicted to result in the loss of function of the gene (Martino et al., 2018). We thus asked whether the bacterial *ackA* was directly involved in the regulation of *PGRP-SC1* expression. To investigate this, we first tested whether *L. plantarum* improvement in fly growth promotion relied on the loss of function of the *ackA* gene by knocking out the *ackA* gene in the ancestor strain *Lp*^{NIZO2877} through CRISPR/Cas9-based bacterial genetic engineering (Jiang et al., 2013). Indeed, we observed that *Lp*$^{\Delta ackA}$ showed a significantly improved growth-promoting effect compared with the ancestor *Lp*^{NIZO2877} ($p < 0.0001$) (Figure 2C). No significant difference was found when comparing *Lp*$^{\Delta ackA}$ and *Lp*$^{FlyG2.1.8}$ and *Lp*$^{DietG20.2.2}$ strains. To test whether the *ackA* loss of function was responsible for the downregulation of the immune response observed in *Lp*$^{FlyG2.1.8}$-associated larvae, we analyzed the expression of *PGRP-SC1* in the gut of larvae associated with *Lp*$^{\Delta ackA}$. Figure 2B shows that the absence of *ackA* resulted in downregulation of the *PGRP-SC1*

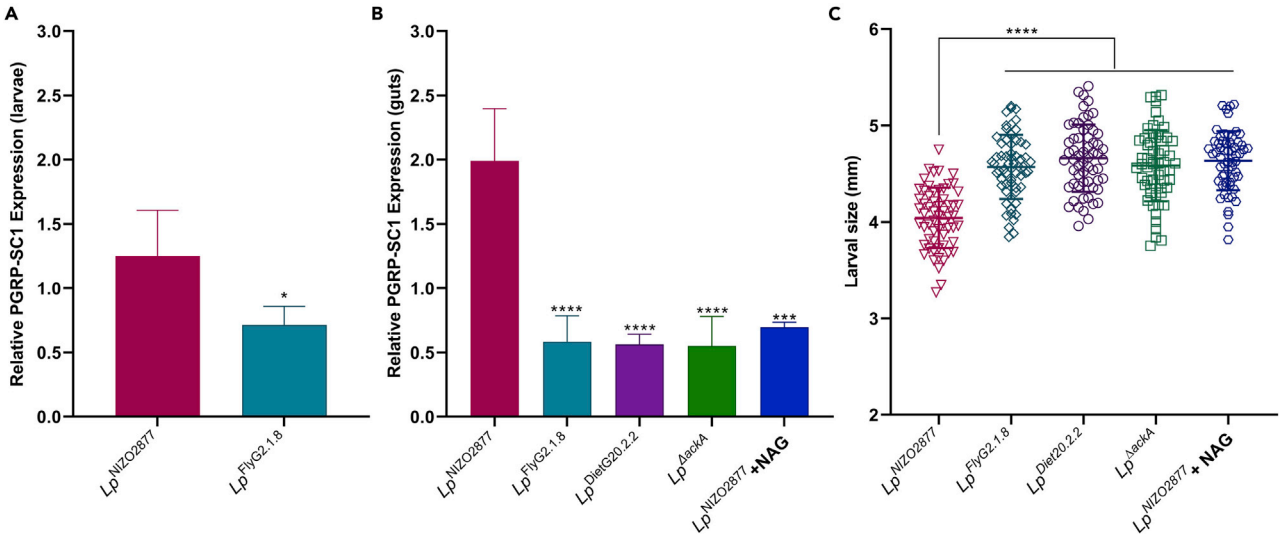

Figure 2. PGRP-SC1 downregulation by beneficial *L. plantarum* strains occurs in Drosophila gut and is regulated by *L. plantarum ackA* function

Relative expression of *PGRP-SC1* gene obtained by performing a qRT-PCR analysis on the transcriptome of *yw Drosophila* larvae (A) and dissected larval guts (B) mono-associated with the bacterial strains tested (x axis). Lines above each bar represent the mean with the standard deviation (SD) calculated by analyzing three biological replicates per condition. Relative expression was calculated as ΔC_T, using the housekeeping gene *rp49* as reference gene. Statistical significance of the results with one-way ANOVA test analysis is included (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

(C) Longitudinal size (mm) of *Drosophila* larvae measured after 7 days of incubation with the bacterial strain $Lp^{NIZO2877}$, $Lp^{FlyG2.1.8}$, $Lp^{DietG20.2.2}$, $Lp^{\Delta ackA}$, and $Lp^{NIZO2877}$ supplemented with N-acetyl glutamine (NAG), respectively. Each symbol refers to the larval size obtained from one out of the N ≥ 60 *Drosophila* larvae analyzed for each condition, with bars referring to the respective mean and standard deviation (SD). All conditions were compared with $Lp^{NIZO2877}$ by performing one-way ANOVA test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

compared with $Lp^{NIZO2877}$-associated larvae. To understand how the bacterial acetate kinase function was involved in Drosophila response against gut microbes, we wondered whether the *L. plantarum* metabolic response resulting from the *ackA* mutation was directly involved in Drosophila immune regulation, or, instead, whether *PGRP-SC1* expression was governed by a more complex mechanism resulting from *ackA* mutation. Specifically, we previously demonstrated that the presence of the $Lp^{FlyG2.1.8}$-*ackA* variant leads to an increase in the bacterial production of N-acetyl-glutamine (NAG), which is sufficient to recapitulate the improved beneficial effect exerted by $Lp^{FlyG2.1.8}$ in the presence of its ancestor (Martino et al., 2018) (Figure 2C). In light of this, we tested the effect of such a metabolite alone on Drosophila *PGRP-SC1* expression by supplementing NAG to $Lp^{NIZO2877}$-associated larvae. Of note, we found that the presence of NAG was sufficient to downregulate *PGRP-SC1* expression in the gut of Drosophila larvae (Figure 2B).

Taken together, our results demonstrate that *PGRP-SC1* expression occurs in Drosophila gut and is regulated by *L. plantarum ackA* function. Specifically, the *ackA* loss of function leads to an increase in the production of N-acetyl-glutamine, which is sufficient to cause *PGRP-SC1* downregulation in Drosophila larvae.

Downregulation of Drosophila PGRP-SC1 is a general response triggered by highly beneficial *L. plantarum* strains

L. plantarum promotes Drosophila growth in a strain-specific manner (Schwarzer et al., 2016; Storelli et al., 2011, 2018). We thus asked whether other beneficial *L. plantarum* strains, phylogenetically distant from $Lp^{FlyG2.1.8}$, improved Drosophila growth by downregulating PGRP-SC1 expression. To do this, we analyzed PGRP-SC1 levels in larvae associated with Lp^{WJL}, a potent *L. plantarum*-growth-promoting strain (Martino et al., 2015a; Storelli et al., 2011) (Figure S3). Lp^{WJL} triggered the downregulation of PGRP-SC1 at the same extent of $Lp^{FlyG2.1.8}$ and the other beneficial strains tested (Figure 3A). Of note, Lp^{WJL} shows two nucleotide substitutions in the *ackA* gene, compared with $Lp^{NIZO2877}$ (Martino et al., 2018), which might concur with its high beneficial effect and the regulation of *Drosophila* immune response. Altogether, our results demonstrate that different highly beneficial *L. plantarum* strains promote Drosophila growth by downregulating the expression of PGRP-SC1 compared with less beneficial strains.

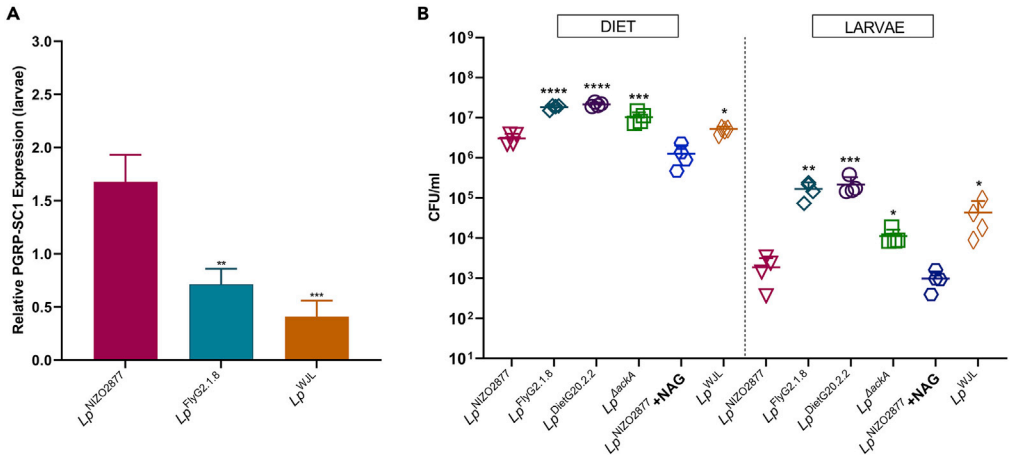

Figure 3. PGRP-SC1 regulation and microbial load

(A) Relative expression of *PGRP-SC1* gene obtained by performing a qRT-PCR analysis on the transcriptome of *Drosophila* larvae mono-associated with *L. plantarum* strains (x axis). Lines above each bar represent the mean with the standard deviation (SD) calculated by analyzing three biological replicates per condition. Relative expression was calculated as ΔC_T, using the housekeeping gene *rp49* as reference gene. Statistical significance of the results with one-way ANOVA test analysis is included:*p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001.

(B) Microbial load (CFU/mL) of the bacterial strains tested after 4 days of incubation in the fly diet and in the intestine of Drosophila larvae, respectively. Each symbol represents one out of the four replicates analyzed for each condition, with bars indicating the respective mean and standard deviation (SD). All conditions were compared with $Lp^{NIZO2877}$ by performing one-way ANOVA test (*p $\leq$ 0.1, **p $\leq$ 0.01, ***p $\leq$ 0.001, ****p $\leq$ 0.0001).

PGRP-SC1 regulation and microbial load

Previous research has shown that *PGRP-SCs* act as negative regulators of IMD pathway activation. Their combined effect shapes the Drosophila antibacterial response and protects the fly from innocuous infections (Bosco-Drayon et al., 2012; Costechareyre et al., 2016; Matos et al., 2018; Paredes et al., 2011). We thus wondered whether the higher expression of *PGRP-SC1* observed in $Lp^{NIZO2877}$-associated larvae resulted in increased bacterial proliferation through negative regulation of the IMD pathway. To do this, we first analyzed the expression of attacin D (*AttD*), a molecular readout of the IMD pathway, and drosomycin (*Drs*), a target gene of Toll-pathway, the second NF-kB signaling cascade involved in fly innate immunity (Choe et al., 2002; Costechareyre et al., 2016; Elrod-Erickson et al., 2000; Kaneko et al., 2004), in larvae associated with $Lp^{NIZO2877}$ and $Lp^{FlyG2.1.8}$. No significant difference was detected between conditions, suggesting that the difference in PGRP-SC1 expression exerted by $Lp^{FlyG2.1.8}$ does not ultimately affect Drosophila normal immunity (Figure S4).

Next, to test if and how PGRP-SC1 levels were correlated with bacterial growth, we quantified the loads of $Lp^{NIZO2877}$, $Lp^{FlyG2.1.8}$, $Lp^{DietG20.2.2}$, $Lp^{\Delta ackA}$, and $Lp^{NIZO2877}$ supplemented with NAG and Lp^{WJL} in the larval gut and in the fly diet at the same time point as the transcriptome analysis (4 days after association). In general, colony counts for *L. plantarum* were significantly lower in larval intestine compared with fly food (Figure 3B). However, we observed that the colony counts for $Lp^{NIZO2877}$ were significantly lower compared with the more beneficial bacterial associations. The only exception was represented by the N-acetylglutamine addition, where *L. plantarum* load reached similar levels both in the fly food and in the fly intestine.

Downregulation of *PGRP-SC* is sufficient to improve Drosophila growth

So far, we have demonstrated that the loss of function of *L. plantarum ackA* gene is responsible for improving the bacterial-growth-promoting effect in Drosophila and that this happens through downregulation of the fly *PGRP-SC1* gene. Following this result, we asked whether the downregulation of *PGRP-SC1* in the larval midgut was sufficient to improve larval growth in response to *L. plantarum*. To test this, we crossed a fly line carrying a UAS-PGRPSC-RNAi construct (Bischoff et al., 2006) with a midgut-specific Mex-Gal4 fly line (driver). In the obtained progeny, the midgut cells produced PGRP-SC-dsRNA targeting the endogenous PGRP-SC transcript to degradation by RNA interference. Mex-Gal4 crossed to

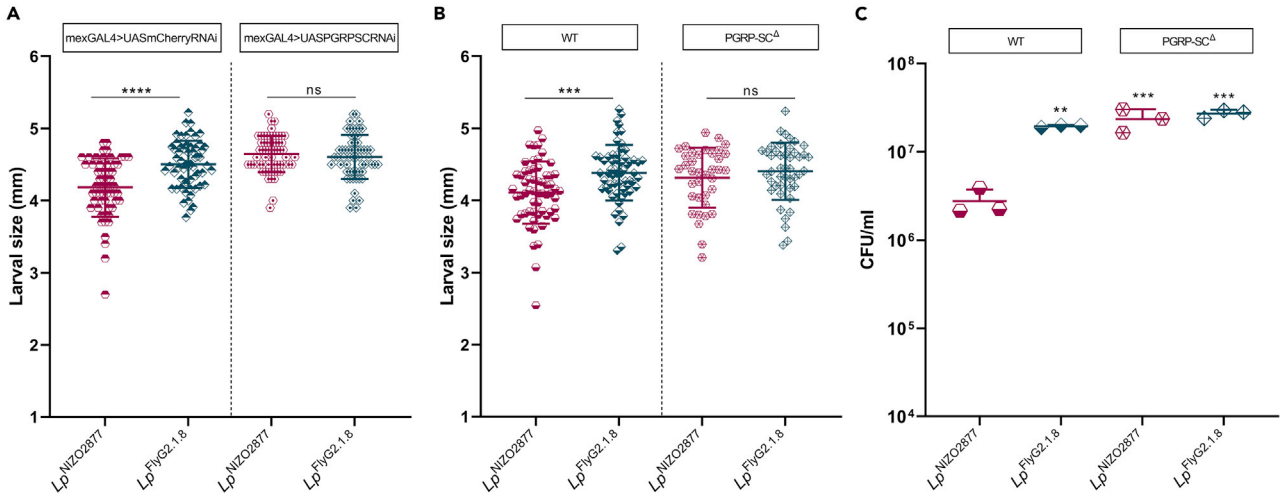

Figure 4. Lp^{NIZO2877} recapitulates the same growth-promoting effect of Lp$^{FlyG2.1.8}$ in mutant PGRP-SC flies

(A) Longitudinal size of Mex-Gal4>UAS-mCherry-RNAi larvae (control) and Mex-Gal4>UAS-PGRPSC-RNAi larvae (target) (A), Oregon-R wild-type (WT) population and larvae carrying a deletion of PGRP-SC gene cluster (PGRP-SC$^{\Delta}$) (B) associated with the bacterial strains *Lp*^{NIZO2877} and *Lp*$^{FlyG2.1.8}$ and measured 7 days after egg deposition on poor-nutrient diet. Each symbol refers to the larval size analyzed for each condition, with bars referring to the respective mean and standard deviation (SD). Each condition was compared with *Lp*^{NIZO2877}-associated larvae of the respective fly background by performing Mann-Whitney test (****p $\leq$ 0.0001, ***p $\leq$ 0.001, ns = not statistically significant).

(C) Microbial load (CFU/mL) of bacteria retrieved from the diet of WT and PGRP-SC$^{\Delta}$ Drosophila larvae associated with the strains *Lp*^{NIZO2877} and *Lp*$^{FlyG2.1.8}$, respectively. Each symbol represents the mean value for each technical replicate, whereas lines are the mean obtained from the values within the same condition with the respective standard deviation (SD). All conditions were compared with WT-*Lp*^{NIZO2877} by performing one-way ANOVA test (*p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001, ****p $\leq$ 0.0001).

UAS-mCherry-RNAi line were used as control. We mono-associated the F1 progeny with *Lp*^{NIZO2877} and *Lp*$^{FlyG2.1.8}$ strains and analyzed their effect on larval growth. Remarkably, we found that, although in control larvae, *Lp*$^{FlyG2.1.8}$ maintained a more marked beneficial effect in promoting larval growth, no difference between the two strains was detected in the Mex-Gal4>UAS-PGRP-SC-RNAi larvae (Figures 4A and S5). To test whether the deletion of the PGRP-SC locus led to *Lp* increase in beneficial effect, we used a mutant fly line bearing a deletion of the *PGRP-SC* gene (*PGRP-SC$^{\Delta}$*) (Paredes et al., 2011). We mono-associated *PGRP-SC$^{\Delta}$* GF embryos with *Lp*^{NIZO2877} and *Lp*$^{FlyG2.1.8}$ strains and analyzed their effect on larval growth compared with axenic conditions in wild-type (WT) larvae (Oregon^{R}). No difference in growth promotion was detected between *Lp*^{NIZO2877}- and *Lp*$^{FlyG2.1.8}$-*PGRP-SC$^{\Delta}$*-associated larvae, whereas *Lp*^{NIZO2877} exhibited a mid-beneficial effect in WT larvae (Figures 4B and S6). Following our previous results showing that *PGRP-SC1* expression is inversely proportional to *Lp*^{NIZO2877}growth (Figure 3B), we then asked whether *Lp*^{NIZO2877} loads were higher in *PGRP-SC$^{\Delta}$* flies than in WT flies. Indeed, we observed that *Lp*^{NIZO2877} reached significantly higher colony counts, with comparable bacterial loads as those of *Lp*$^{FlyG2.1.8}$ in both the mutant and WT lines (Figure 4C). Altogether, our results show that *L. plantarum* is able to improve Drosophila growth by regulating its immune response via the downregulation of *PGRP-SC1*, which is associated with increased bacterial growth and proliferation.

DISCUSSION

In the present study, we aimed at understanding the Drosophila transcriptional pathways regulated by beneficial gut commensals and leading to improved growth. Consistent with previous work, we showed that gut microbes strongly affect host's gene expression (Nichole A. Broderick et al., 2014; Combe et al., 2014; Dobson et al., 2016), and such effects happen regardless of their benefit potential. Indeed, the presence of two isogenic *L. plantarum* strains differing in growth promotion significantly altered gene expression in Drosophila compared with axenic conditions. Analysis of the genes differentially expressed between gnotobiotic and axenic conditions revealed the upregulation of genes related to host immune response in the presence of bacteria (Figure S2, Tables S1 and S2). It is well known that commensal bacteria are basal inducers of host immune responses (Broderick et al., 2014; Buchon et al., 2009, 2013; Combe et al., 2014; Erkosar et al., 2012; Ryu et al., 2008). Our analysis highlighted the significant impact of peptidoglycan-recognition proteins (e.g., *PGRP-SB1*, *PGRP-SD*, *PGRP-LA*, *PGRP-SCs*) in Drosophila

immune system regulation and response to bacteria. PGRPs represent the main and most diverse functional family of peptidoglycan detection proteins (Anselme et al., 2006; Dziarski and Gupta, 2010; Li et al., 2007; Royet and Dziarski, 2007; Troll et al., 2010; Yu et al., 2010) and have been largely reported as crucial effectors in regulating the antibacterial response in Drosophila (Buchon et al., 2014; Kurata, 2010; Royet et al., 2011). We also detected an enriched expression of lysozyme genes (i.e., *LysB, C, D* and *E*), which are synthesized at a high rate in the midgut of larvae and adult flies and are known to have a specific role in the digestive process (Daffre et al., 1994; Marra et al., 2021). In addition, they are involved in the hydrolysis of beta-linkages between *N-acetylmuramic* acid and *N-acetyl-D-glucosamine* residues in bacterial peptidoglycans, likely supporting the action of the PGRP-SC genes in the antibacterial response (Nash et al., 2006). In addition to immune system regulation, bacterial association caused the upregulation of genes known to be involved in stress response (e.g., *hsp23*, *GstD8*), proteolysis (*Jon66ci*, *Jon66cii*, *Jon44E*, *Jon99ci*, *Bace*, etc.), and metabolism (Figure S2, Tables S1 and S2). All these results are largely in agreement with those of previous studies examining the impact of gut commensals on *D. melanogaster* transcriptional regulation across different fly developmental stages and populations (Nichole A Broderick et al., 2014; Combe et al., 2014).

We next sought to determine the host genes specifically altered by the presence of commensal bacteria that promote Drosophila growth and development. To do this, we compared the transcriptional response of Drosophila larvae associated with two isogenic *L. plantarum* strains: a strong growth-promoting strain ($Lp^{FLYG2.1.8}$) and the mid-beneficial strain $Lp^{NIZO2877}$. We detected differential expression of only 21 genes between the two conditions. Specifically, $Lp^{FLYG2.1.8}$ led to a strong upregulation of the Sperm-Leucylaminopeptidase 3 (S-lap3) compared with $Lp^{NIZO2877}$-associated larvae, reaching a variation of 23-fold. Sperm-Leucylaminopeptidases have been described as structural components of the paracrystalline material of Drosophila sperm, and they are known to be expressed exclusively during spermatogenesis (Dorus et al., 2011; Laurinyecz et al., 2019). However, although gonadal development begins during larval and pupal stages, Drosophila larvae are not sexually mature (Markow, 2002, 1988; Markow and O'Grady, 2008). Here, we do not expect that the S-lap regulation is directly linked to fly fertility or sperm function. Instead, being peptidases, they may be involved in host development and maturation; this is in line with the upregulation of protease genes that have been detected in the presence of $Lp^{FLYG2.1.8}$ (e.g., CG43124, CG43125). Beneficial microbes have been directly associated with increased proteolytic activities, which improve host nutrition and lead to faster development (Combe et al., 2014; Erkosar et al., 2012). Here, we speculate that the upregulation of Sperm-Leucylaminopeptidases exerted by $Lp^{FLYG2.1.8}$ might impact larval nutrition and development, which is known to affect the subsequent adult reproductive success (Robertson, 1954, 1957). Indeed, $Lp^{FLYG2.1.8}$ also triggers the upregulation of genes directly involved in Drosophila development and lifespan (i.e., *mthl6*) and nutrient sensing (*Obp56e*). *mthl6* belongs to the Methuselah/Methuselah-like (Mth/Mthl) gene family of G-protein-coupled receptors, which are essential for larval development, stress resistance, and in the setting of adult lifespan (Araújo et al., 2013; De Mendoza et al., 2016; Lin et al., 1998). On the other hand, the odorant-binding protein *Obp56e* is required for the detection of several essential amino acids. Its upregulation in the presence of $Lp^{FLYG2.1.8}$ might be thus linked to a higher efficiency in nutrient sensing and assimilation as well as potentially higher food intake. Further work would be needed to investigate the actual role of S-lap family in larval maturation and growth and the consequences of altered expression on fly fertility.

The most surprising finding of this study was the downregulation of the immune response exerted by the beneficial strain $Lp^{FLYG2.1.8}$. Although both $Lp^{FLYG2.1.8}$ and $Lp^{NIZO2877}$ strains cause an increased expression of peptidoglycan recognition proteins compared with axenic larvae, the association with $Lp^{FLYG2.1.8}$ resulted in the specific lower expression of *PGRP-SC1a* and *PGRP-SC1b* compared with $Lp^{NIZO2877}$-associated larvae. The PGRP-SC gene cluster contains three PGRP-SC isoforms: PGRP-SC1a, PGRP-SC1b, and PGRP-SC2. PGRP-SC1a and PGRP-SC1b probably arose from a recent duplication, given that the two genes differ only by a synonymous mutation and produce the same polypeptide (Paredes et al., 2011). They display an amidasic activity, as they can bind and hydrolyze the bacterial peptidoglycan into smaller nonimmunogenic muropeptides (Costechareyre et al., 2016; Mellroth and Steiner, 2006; Zaidman-Rémy et al., 2006, 2011). In contrast, noncatalytic PGRPs (i.e., PGRP-SA, PGRP-SD, PGRP-LC, PGRP-LE, etc.) bind to peptidoglycan but lack amidase activity. They are essential sentinels upstream of the two NF-κB-dependent *Drosophila* signaling cascades called Toll and immune deficiency (IMD) (Kurata, 2010). Although the mode of action of noncatalytic PGRPs in the Drosophila immune response and bacterial recognition has been extensively studied (Bischoff et al., 2004; Choe et al., 2002, 2005; Gottar et al.,

2002; Kaneko et al., 2006; Leone et al., 2008; Michel et al., 2001; Rämet et al., 2002; Takehana et al., 2004; Wang et al., 2008; Werner et al., 2003), the precise role of PGRP-SCs is less well defined. In this context, few studies have focused on determining the function of PGRP-SCs. Specifically, the amidasic activity of PGRP-SCs has been shown to result in dampening the IMD pathway, which appears to be under strong negative regulation in flies (Bischoff et al., 2006; Costechareyre et al., 2016; Paredes et al., 2011); this is known as immune tolerance, and it is meant to prevent constitutive activation of the energy-consuming NF-kB pathways (Grenier and Leulier, 2020). In addition, it has been demonstrated that PGRP-SC1 and PGRP-SC2 are required *in vivo* for full Toll pathway activation by Gram-positive bacteria and for phagocytosis (Costechareyre et al., 2016; Garver et al., 2006). Here, we reveal that highly beneficial *L. plantarum* strains are able to exert their growth-promoting effect by specifically downregulating PGRP-SC1 compared with less beneficial strains, with no effect on other PGRPs. First, we hypothesized that such effect might be driven by the evolutionary history of $Lp^{FLYG2.1.8}$, which has been co-evolving with the fly for two generations (Martino et al., 2018). However, our testing of other beneficial strains with different evolutionary backgrounds (i.e., $Lp^{DietG20.2.2}$) or phylogenetically distant from $Lp^{FLYG2.1.8}$ (i.e., Lp^{WJL}) led to the same effect in *PGRP-SC1* transcriptional regulation (Figures 2A and 3A); this suggests that PGRP-SC1 regulation by growth-promoting *L. plantarum* strains is not strain specific but might reflect a general mechanism of microbe-mediated growth promotion.

We demonstrated that the lower expression of PGRP-SC1 directly results from the loss of function of the *L. plantarum ackA* gene. Indeed, we show that deleterious mutations in the *ackA* gene of different *L. plantarum* strains cause PGRP-SC1 downregulation, which in turns leads to the improvement of the *L. plantarum* growth-promoting effect. It is interesting to note that the lower expression of PGRP-SC1 does not result from decreased bacterial loads in the food or in the larval intestine. Contrarily, all *L. plantarum* strains bearing the *ackA* mutation exhibited higher loads compared with $Lp^{NIZO2877}$. At the same time, we show that the bacterial metabolic rewiring following *ackA* mutation is sufficient to downregulate the expression of PGRP-SC1. In particular, the supplementation of N-acetyl-glutamine, a direct consequence of *ackA* loss of function, was sufficient to trigger the lower expression of PGRP-SC1 without causing an increase in bacterial colony counts; this suggests that increasing NAG concentration in the presence of a mid-beneficial strain is able to recapitulate the effect triggered by higher bacterial loads (i.e., downregulation of PGRP-SC1 and improved host growth promotion), but it is not sufficient to increase bacterial growth *per se*. We believe that other metabolic pathways separate from those that increased NAG production are related to the improved growth of *L. plantarum* in association with Drosophila.

We next asked whether the specific downregulation of PGRP-SC in the larval midgut, as well as the deletion of the PGRP-SC cluster, were sufficient to improve the beneficial effect of $Lp^{NIZO2877}$. Remarkably, we found that $Lp^{NIZO2877}$ significantly improves its benefit, recapitulating the effect exerted by $Lp^{FLYG2.1.8}$, both in Mex-Gal4>UAS-PGRP-SC-RNAi and $PGRP\text{-}SC^{\Delta}$ flies. Importantly, we show that such improvement in growth promotion likely results from the higher bacterial loads reached by $Lp^{NIZO2877}$, compared with the reference population (Figure 4C). Again, this result points out the importance of bacterial colony counts in affecting larval growth and development. Altogether, our data demonstrate that the degree of benefit exerted by *L. plantarum* toward Drosophila relies on the regulation of its immune response. Specifically, highly beneficial *L. plantarum* strains improve Drosophila growth by downregulating the expression of PGRP-SC1 in the larval midgut compared with moderate beneficial strains. The downregulation of such peptidoglycan catalytic enzymes is likely associated with a decreased bactericidal activity and higher viable bacterial cells, which in turn benefit larval development and maturation. It is important to note that we did not detect any significant difference in the regulation of the IMD pathway (i.e., *pirk*, *Rel*, *AttD* gene expression) nor the Toll pathway (i.e., *Drs* expression) between $Lp^{FLYG2.1.8}$- and $Lp^{NIZO2877}$-associated larvae (Table S1 and Figure S4), indicating that the higher expression of *PGRP-SC1* observed in the presence of $Lp^{NIZO2877}$ does not result in the IMD negative regulation, as expected from previous works (Bischoff et al., 2006; Costechareyre et al., 2016). On the other hand, PGRP-SC downregulation following $Lp^{FLYG2.1.8}$ higher loads both in the medium and in the larval leads to improved host growth, further proving that bacterial concentration is directly proportional to microbial benefit; this is in line with the dual effect that commensal bacteria exert on their host. First, Drosophila larvae can use the microbial biomass as a source of additional nutrients, especially under nutrient-scarce conditions (Keebaugh et al., 2018; Storelli et al., 2018). Secondly, live microbes can improve animal metabolism and amino acid absorption by increasing the host's intestinal peptidases activity shown here and in prior work (Dziarski and Gupta, 2010; Royet and Dziarski, 2007). Moreover, live microbes actively produce and release essential nutrients (Consuegra

et al., 2019, 2020a; Martino et al., 2018; Storelli et al., 2018) whose density arises as bacterial loads increase. In this light, we specifically demonstrated that the increase in the production of metabolic products of live bacteria (i.e., N-acetyl-glutamine), which mimics the effect of higher microbial loads, is sufficient to trigger a lower expression of PGRP-SC1 and improve larval growth. The effect of bacterial metabolites on the regulation of Drosophila immune system has been also reported in previous studies (Kamareddine et al., 2018; Shin et al., 2011). Kamareddine et al. demonstrated that acetate produced by commensal bacteria from the *Acetobacter* genus can alter the expression of IMD pathway genes through PGRP-LC regulation in the enteroendocrine cells, which leads to mobilization of lipid resources in the nearby enterocytes and ultimately to growth promotion (Kamareddine et al., 2018). Although these data show that local signaling of bacterial by-products have systemic consequences on the host's development, it remains unknown how such metabolites (i.e., N-acetyl-glutamine, acetate, etc.) directly affect Drosophila immune response.

Collectively, our work shows that the lower expression of PGRP-SC1 in Drosophila midgut caused by beneficial commensal bacteria is directly responsible of improved larval growth. PGRPs are highly conserved from insects to mammals. Although, in insects, PGRPs are mostly involved in regulating defense pathways, in mammals, they have primarily antimicrobial activities (Gelius et al., 2003; Lu et al., 2006; Paredes et al., 2011; Wang et al., 2007). Here, we show that a lower expression of PGRP-SC1 is directly linked to higher microbial loads, suggesting the specific bactericidal activity of such protein, with no effect on the regulation of immune pathways; this demonstrates that intestinal immune tolerance mechanisms to beneficial bacteria are critical to lead to higher bacterial growth and thus to improved host's health. Signaling through immune pathways happens via the activity of bacterial peptidoglycan and metabolic products (i.e., N-acetyl-glutamine), which are directly responsible of host immune regulation and growth. Our results reinforce the notion that the influence of beneficial microbes on Drosophila physiology therefore relies on the intricate network of nutritional, metabolic, and immune inputs (Grenier and Leulier, 2020). Further work would be needed to dissect the interdependency of such processes and to reveal the mechanisms underlying the bacterial specificity toward Drosophila immune effectors.

Limitations of the study

This study shows that beneficial commensal bacteria improve larval growth by downregulating the expression of PGRP-SC in the larval midgut. Moreover, such transcriptional regulation is caused by the loss of function of *L. plantarum ackA* gene and the increase of N-acetyl-amino acids. However, future studies are required to reveal how bacterial acetate metabolism influence Drosophila immune response. In addition, in this study we employed Drosophila transgenic lines bearing either the downregulation or the deletion of PGRP-SC cluster. We have not been able to employ the genomic rescue of the PGRP-SCs deficiency lines or create Drosophila transgenic lines bearing the specific downregulation of PGRP-SC1 to verify that bacterial proliferation triggers the lower expression of PGRP-SC1 in larval midgut. It will be thus interesting to test the effect of *L. plantarum* on Drosophila lines bearing the mutation of PGRP-SC1 alone; this will allow to further proving the specificity of our results.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104357.

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AUTHOR CONTRIBUTIONS

M.G. and M.E.M designed the project; M.G., E.M., M.B., and N.S. conducted the experiments; P.J. and A.Q. conducted the bioinformatics analyses; J.V and C.B. designed and performed the CRISPR-Cas9 editing experiments; M.G. and M.E.M analyzed the data and wrote the paper.

DECLARATION OF INTERESTS

The authors declared no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

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REFERENCES

Anselme, C., Vallier, A., Balmand, S., Fauvarque, M.O., and Heddi, A. (2006). Host PGRP gene expression and bacterial release in endosymbiosis of the weevil Sitophilus zeamais. Appl. Environ. Microbiol. *72*, 6766–6772. https://doi.org/10.1128/AEM.00942-06.

Araújo, A.R., Reis, M., Rocha, H., Aguiar, B., Morales-Hojas, R., Macedo-Ribeiro, S., Fonseca, N.A., Reboiro-Jato, D., Reboiro-Jato, M., Fdez-Riverola, F., et al. (2013). The Drosophila melanogaster methuselah gene: a novel gene with ancient functions. PLoS One *8*, 1–12. https://doi.org/10.1371/journal.pone.0063747.

Bischoff, V., Vignal, C., Boneca, I.G., Michel, T., Hoffmann, J.A., and Royet, J. (2004). Function of the drosophila pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria. Nat. Immunol. *5*, 1175–1180. https://doi.org/10.1038/ni1123.

Bischoff, V., Vignal, C., Duvic, B., Boneca, I.G., Hoffmann, J.A., and Royet, J. (2006). Downregulation of the Drosophila immune response by peptidoglycan- recognition proteins SC1 and SC2. PLoS Pathog. *2*, e14. https://doi.org/10.1371/journal.ppat.0020014.

Blum, J.E., Fischer, C.N., Miles, J., and Handelsman, J. (2013). Frequent replenishment sustains the beneficial microbiome of Drosophila melanogaster. MBio *4*, 1–8. https://doi.org/10.1128/mBio.00860-13.

Bosco-Drayon, V., Poidevin, M., Boneca, I.G., Narbonne-Reveau, K., Royet, J., and Charroux, B. (2012). Peptidoglycan sensing by the receptor PGRP-LE in the Drosophila gut induces immune responses to infectious bacteria and tolerance to microbiota. Cell Host Microbe *12*, 153–165. https://doi.org/10.1016/j.chom.2012.06.002.

Bost, A., Franzenburg, S., Adair, K.L., Martinson, V.G., Loeb, G., and Douglas, A.E. (2018). How gut transcriptional function of Drosophila melanogaster varies with the presence and composition of the gut microbiota. Mol. Ecol. *27*, 1848–1859. https://doi.org/10.1111/mec.14413.

Broderick, Nichole A., Buchon, N., and Lemaitre, B. (2014). Microbiota-induced changes in Drosophila melanogaster host gene expression and gut morphology. MBio *5*, 1–13. https://doi.org/10.1128/mBio.01117-14.

Brummel, T., Ching, A., Seroude, L., Simon, A.F., and Benzer, S. (2004). Drosophila lifespan enhancement by exogenous bacteria. Proc. Natl. Acad. Sci. U S A *101*, 12974–12979. https://doi.org/10.1073/pnas.0405207101.

Buchon, N., Broderick, N.A., Chakrabarti, S., and Lemaitre, B. (2009). Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila. Genes Dev. *23*, 2333–2344. https://doi.org/10.1101/gad.1827009.

Buchon, N., Broderick, N.A., and Lemaitre, B. (2013). Gut homeostasis in a microbial world: insights from Drosophila melanogaster. Nat. Rev. Microbiol. *11*, 615–626. https://doi.org/10.1038/nrmicro3074.

Buchon, N., Silverman, N., and Cherry, S. (2014). Immunity in Drosophila melanogaster-from microbial recognition to whole-organism physiology. Nat. Rev. Immunol. *14*, 796–810. https://doi.org/10.1038/nri3763.

Chandler, J.A., Lang, J., Bhatnagar, S., Eisen, J.A., and Kopp, A. (2011). Bacterial communities of diverse Drosophila species: ecological context of a host-microbe model system. PLoS Genet. *7*, e1002272. https://doi.org/10.1371/journal.pgen.1002272.

Choe, K.M., Lee, H., and Anderson, K.V. (2005). Drosophila peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor. Proc. Natl. Acad. Sci. U S A *102*, 1122–1126. https://doi.org/10.1073/pnas.0404952102.

Choe, K.M., Werner, T., Stöven, S., Hultmark, D., and Anderson, K.V. (2002). Requirement for a peptidoglycan recognition protein (PGRP) in relish activation and antibacterial immune responses in Drosophila. Science *296*, 359–362. https://doi.org/10.1126/science.1070216.

Combe, B.E., Defaye, A., Bozonnet, N., Puthier, D., Royet, J., and Leulier, F. (2014). Drosophila microbiota modulates host metabolic gene expression via IMD/NF-κB signaling. PLoS One *9*, e94729. https://doi.org/10.1371/journal.pone.0094729.

Consuegra, J., Grenier, T., Akherraz, H., Rahioui, I., Gervais, H., da Silva, P., and Leulier, F. (2020a). Metabolic cooperation among commensal bacteria supports Drosophila juvenile growth under nutritional stress. iScience *23*, 101232. https://doi.org/10.1016/j.isci.2020.101232.

Consuegra, J., Grenier, T., Baa-Puyoulet, P., Rahioui, I., Akherraz, H., Gervais, H., Parisot, N., da Silva, P., Charles, H., Calevro, F., and Leulier, F. (2019). Commensal bacteria differentially shape the nutritional requirements of Drosophila during juvenile growth. PLoS Biol. *18*, e3000681. https://doi.org/10.1101/728774.

Consuegra, J., Grenier, T., Baa-Puyoulet, P., Rahioui, I., Akherraz, H., Gervais, H., Parisot, N., Da Silva, P., Charles, H., Calevro, F., and Leulier, F. (2020b). Drosophila-associated bacteria differentially shape the nutritional requirements of their host during juvenile growth. PLoS Biol. *18*, e3000681. https://doi.org/10.1371/journal.pbio.3000681.

Costechareyre, D., Capo, F., Fabre, A., Chaduli, D., Kellenberger, C., Roussel, A., Charroux, B., and Royet, J. (2016). Tissue-specific regulation of Drosophila NF-κB pathway activation by peptidoglycan recognition protein SC. J. Innate Immun. *8*, 67–80. https://doi.org/10.1159/000437368.

Daffre, S., Kylsten, P., Samakovlis, C., and Hultmark, D. (1994). The lysozyme locus in Drosophila melanogaster: an expanded gene family adapted for expression in the digestive tract. MGG Mol. Gen. Genet. *242*, 152–162. https://doi.org/10.1007/BF00391008.

De Mendoza, A., Jones, J.W., and Friedrich, M. (2016). Methuselah/Methuselah-like G protein-coupled receptors constitute an ancient metazoan gene family. Sci. Rep. *6*, 21801. https://doi.org/10.1038/srep21801.

Delbare, S.Y.N., Ahmed-Braimah, Y.H., Wolfner, M.F., and Clark, A.G. (2020). Interactions between the microbiome and mating influence the female's transcriptional profile in Drosophila melanogaster. Sci. Rep. *10*, 18168. https://doi.org/10.1038/s41598-020-75156-9.

Dobson, A.J., Chaston, J.M., and Douglas, A.E. (2016). The Drosophila transcriptional network is structured by microbiota. BMC Genom. *17*, 975–979. https://doi.org/10.1186/s12864-016-3307-9.

Dorus, S., Wilkin, E.C., and Karr, T.L. (2011). Expansion and functional diversification of a leucyl aminopeptidase family that encodes the major protein constituents of Drosophila sperm. BMC Genom. *12*, 177. https://doi.org/10.1186/1471-2164-12-177.

Douglas, A.E. (2011). Lessons from studying insect symbioses. Cell Host Microbe *10*, 359–367. https://doi.org/10.1016/j.chom.2011.09.001.

Dziarski, R., and Gupta, D. (2010). Review: mammalian peptidoglycan recognition proteins (PGRPs) in innate immunity. Innate Immun. *16*, 168–174. https://doi.org/10.1177/1753425910366059.

Elrod-Erickson, M., Mishra, S., and Schneider, D. (2000). Interactions between the cellular and humoral immune responses in Drosophila. Curr. Biol. *10*, 781–784. https://doi.org/10.1016/S0960-9822(00)00569-8.

Erkosar, B., Storelli, G., Defaye, A., and Bernard, C. (2012). " Learning on the Fly " (13: Cell Host Microbe), pp. 8–14. https://doi.org/10.1016/j.chom.2012.12.004.

Erkosar, B., Storelli, G., Defaye, A., and Leulier, F. (2013). Host-intestinal microbiota mutualism: "learning on the fly. Cell Host Microbe *13*, 8–14. https://doi.org/10.1016/j.chom.2012.12.004.

Erkosar, B., Storelli, G., Mitchell, M., Bozonnet, L., Bozonnet, N., and Leulier, F. (2015). Pathogen virulence impedes Mutualist-mediated enhancement of host juvenile growth via inhibition of protein digestion. Cell Host Microbe *18*, 445–455. https://doi.org/10.1016/j.chom.2015.09.001.

Fraune, S., Anton-Erxleben, F., Augustin, R., Franzenburg, S., Knop, M., Schröder, K., Willoweit-Ohl, D., and Bosch, T.C.G. (2015). Bacteria-bacteria interactions within the microbiota of the ancestral metazoan Hydra contribute to fungal resistance. ISME J. *9*, 1543–1556. https://doi.org/10.1038/ismej.2014.239.

Fraune, S., and Bosch, T.C.G. (2010). Why bacteria matter in animal development and evolution. Bioessays *32*, 571–580. https://doi.org/10.1002/bies.200900192.

Garver, L.S., Wu, J., and Wu, L.P. (2006). The peptidoglycan recognition protein PGRP-SC1a is essential for Toll signaling and phagocytosis of Staphylococcus aureus in Drosophila. Proc. Natl. Acad. Sci. U S A *103*, 660–665. https://doi.org/10.1073/pnas.0506182103.

Gelius, E., Persson, C., Karlsson, J., and Steiner, H. (2003). A mammalian peptidoglycan recognition protein with N-acetylmuramoyl-L-alanine amidase activity. Biochem. Biophys. Res. Commun. *306*, 988–994. https://doi.org/10.1016/S0006-291X(03)01096-9.

Gilbert, J.A., and Neufeld, J.D. (2014). Life in a world without microbes. PLoS Biol. *12*, 1–3. https://doi.org/10.1371/journal.pbio.1002020.

Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J.A., Ferrandon, D., and Royet, J. (2002). The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature *416*, 640–644. https://doi.org/10.1038/nature734.

Grenier, T., and Leulier, F. (2020). How commensal microbes shape the physiology of Drosophila melanogaster. Curr. Opin. Insect Sci. *41*, 92–99. https://doi.org/10.1016/j.cois.2020.08.002.

Guo, L., Karpac, J., Tran, S.L., and Jasper, H. (2014). PGRP-SC2 promotes gut immune homeostasis to limit commensal dysbiosis and extend lifespan. Cell *156*, 109–122. https://doi.org/10.1016/j.cell.2013.12.018.

Hooper, L.V., Midwedt, T., and Gordon, J.I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu. Rev. Nutr. *22*, 283–307. https://doi.org/10.1146/annurev.nutr.22.011602.092259.

Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. *4*, 44–57. https://doi.org/10.1038/nprot.2008.211.

Jiang, W., Bikard, D., Cox, D., Zhang, F., and Marraffini, L.A. (2013). RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat. Biotechnol. *31*, 233–239. https://doi.org/10.1038/nbt.2508.

Kamareddine, L., Robins, W.P., Berkey, C.D., Mekalanos, J.J., and Watnick, P.I. (2018). The Drosophila immune deficiency pathway modulates enteroendocrine function and host metabolism. Cell Metab. *28*, 449–462.e5. https://doi.org/10.1016/j.cmet.2018.05.026.

Kaneko, T., Goldman, W.E., Mellroth, P., Steiner, H., Fukase, K., Kusumoto, S., Harley, W., Fox, A., Golenbock, D., and Silverman, N. (2004). Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway. Immunity *20*, 637–649. https://doi.org/10.1016/S1074-7613(04)00104-9.

Kaneko, T., Yano, T., Aggarwal, K., Lim, J.H., Ueda, K., Oshima, Y., Peach, C., Erturk-Hasdemir, D., Goldman, W.E., Oh, B.H., et al. (2006). PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. Nat. Immunol. *7*, 715–723. https://doi.org/10.1038/ni1356.

Karunakar, P., Bhalla, A., and Sharma, A. (2019). Transgenerational inheritance of cold temperature response in Drosophila. FEBS Lett. *593*, 594–600. https://doi.org/10.1002/1873-3468.13343.

Keebaugh, E.S., Yamada, R., Obadia, B., Ludington, W.B., and Ja, W.W. (2018). Microbial quantity impacts Drosophila nutrition, development, and lifespan. iScience *4*, 247–259. https://doi.org/10.1016/j.isci.2018.06.004.

Kurata, S. (2010). Extracellular and intracellular pathogen recognition by Drosophila PGRP-LE and PGRP-LC. Int. Immunol. *22*, 143–148. https://doi.org/10.1093/intimm/dxp128.

Laurinyecz, B., Vedelek, V., Kovács, A.L., Szilasi, K., Lipinszki, Z., Slezák, C., Darula, Z., Juhász, G., and Sinka, R. (2019). Sperm-Leucylaminopeptidases are required for male fertility as structural components of mitochondrial paracrystalline material in Drosophila melanogaster sperm. PLoS Genet. *15*,

1–24. https://doi.org/10.1371/journal.pgen.1007987.

Lee, W.J., and Brey, P.T. (2013). How microbiomes influence metazoan development: insights from history and drosophila modeling of gut-microbe interactions. Annu. Rev. Cell Dev. Biol. *29*, 571–592. https://doi.org/10.1146/annurev-cellbio-101512-122333.

Leenay, R.T., Vento, J.M., Shah, M., Martino, M.E., Leulier, F., and Beisel, C.L. (2019). Genome editing with CRISPR-cas9 in Lactobacillus plantarum revealed that editing outcomes can vary across strains and between methods. Biotechnol. J. *14*, e11700583. https://doi.org/10.1002/biot.201700583.

Leone, P., Bischoff, V., Kellenberger, C., Hetru, C., Royet, J., and Roussel, A. (2008). Crystal structure of Drosophila PGRP-SD suggests binding to DAP-type but not lysine-type peptidoglycan. Mol. Immunol. *45*, 2521–2530. https://doi.org/10.1016/j.molimm.2008.01.015.

Lhocine, N., Ribeiro, P.S., Buchon, N., Wepf, A., Wilson, R., Tenev, T., Lemaitre, B., Gstaiger, M., Meier, P., and Leulier, F. (2008). PIMS modulates immune tolerance by negatively regulating Drosophila innate immune signaling. Cell Host Microbe *4*, 147–158. https://doi.org/10.1016/j.chom.2008.07.004.

Li, X., Wang, S., Qi, J., Echtenkamp, S.F., Chatterjee, R., Wang, M., Boons, G.J., Dziarski, R., and Gupta, D. (2007). Zebrafish peptidoglycan recognition proteins are bactericidal amidases essential for defense against bacterial infections. Immunity *27*, 518–529. https://doi.org/10.1016/j.immuni.2007.07.020.

Lin, Y.J., Seroude, L., and Benzer, S. (1998). Extended life-span and stress resistance in the Drosophila mutant methuselah. Science *282*, 943–946. https://doi.org/10.1126/science.282.5390.943.

Lu, X., Wang, M., Qi, J., Wang, H., Li, X., Gupta, D., and Dziarski, R. (2006). Peptidoglycan recognition proteins are a new class of human bactericidal proteins. J. Biol. Chem. *281*, 5895–5907. https://doi.org/10.1074/jbc.M511631200.

Ma, D., Storelli, G., Mitchell, M., and Leulier, F. (2015). Studying host-microbiota mutualism in Drosophila: harnessing the power of gnotobiotic flies. Biomed. J. *38*, 285–293. https://doi.org/10.4103/2319-4170.158620.

Markow, T.A. (1988). Reproductive behavior of Drosophila melanogaster and D. nigrospiracula in the field and in the laboratory. J. Comp. Psychol. *102*, 169–173. https://doi.org/10.1037/0735-7036.102.2.169.

Markow, T.A. (2002). Perspective: female remating, operational sex ratio, and the arena of sexual selection in Drosophila species. Evolution (N. Y). *56*, 1725–1734. https://doi.org/10.1111/j.0014-3820.2002.tb00186.x.

Markow, T.A., and O'Grady, P. (2008). Reproductive ecology of Drosophila. Funct. Ecol. *22*, 747–759. https://doi.org/10.1111/j.1365-2435.2008.01457.x.

Marra, A., Hanson, M.A., Kondo, S., Erkosar, B., and Lemaitre, B. (2021). Drosophila antimicrobial peptides and lysozymes regulate gut microbiota composition and abundance. Preprint at bioRxiv. https://doi.org/10.1128/mBio.00824-21.

Martino, M.E., Bayjanov, J.R., Joncour, P., Hughes, S., Gillet, B., Kleerebezem, M., Siezen, R., van Hijum, S.A.F.T., and Leulier, F. (2015a). Nearly complete genome sequence of Lactobacillus plantarum strain NIZO2877. Genome Announc. *3*, e01370-15. https://doi.org/10.1128/genomeA.01370-15.

Martino, M.E., Bayjanov, J.R., Joncour, P., Hughes, S., Gillet, B., Kleerebezem, M., Siezen, R., van Hijum, S.A.F.T., and Leulier, F. (2015b). Resequencing of the Lactobacillus plantarum strain WJL genome. Genome Announc. *3*, 5242. https://doi.org/10.1128/genomeA.01382-15.

Martino, M.E., Joncour, P., Leenay, R., Gervais, H., Shah, M., Hughes, S., Gillet, B., Beisel, C., and Leulier, F. (2018). Bacterial adaptation to the host's diet is a key evolutionary force shaping drosophila-Lactobacillus symbiosis. Cell Host Microbe *24*, 109–119. https://doi.org/10.1016/j.chom.2018.06.001.

Matos, R.C., Schwarzer, M., Gervais, H., Courtin, P., Joncour, P., Gillet, B., Ma, D., Bulteau, A.L., Martino, M.E., Hughes, S., et al.; Europe PMC Funders Group (2018). D-alanine esterification of teichoic acids contributes to Lactobacillus plantarum mediated Drosophila growth promotion upon chronic undernutrition. Nat. Microbiol. *2*, 1635–1647. https://doi.org/10.1038/s41564-017-0038-x.

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C.G., Carey, H.V., Domazet-Lošo, T., Douglas, A.E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S.F., et al. (2013). Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci. U S A *110*, 3229–3236. https://doi.org/10.1073/pnas.1218525110.

Mellroth, P., and Steiner, H. (2006). PGRP-SB1: an N-acetylmuramoyl l-alanine amidase with antibacterial activity. Biochem. Biophys. Res. Commun. *350*, 994–999. https://doi.org/10.1016/j.bbrc.2006.09.139.

Michel, T., Reichhart, J.M., Hoffmann, J.A., and Royet, J. (2001). Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature *414*, 756–759. https://doi.org/10.1038/414756a.

Nash, J.A., Ballard, T.N.S., Weaver, T.E., and Akinbi, H.T. (2006). The peptidoglycan-degrading property of lysozyme is not required for bactericidal activity *in vivo*. J. Immunol. *177*, 519–526. https://doi.org/10.4049/jimmunol.177.1.519.

Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., and Pettersson, S. (2012). Host-gut microbiota metabolic interactions. Science *336*, 1262–1267. https://doi.org/10.1126/science.1223813.

Paredes, J.C., Welchman, D.P., Poidevin, M., and Lemaitre, B. (2011). Negative regulation by Amidase PGRPs shapes the drosophila antibacterial response and protects the Fly from innocuous infection. Immunity *35*, 770–779. https://doi.org/10.1016/j.immuni.2011.09.018.

Phillips, M.D., and Thomas, G.H. (2006). Brush border spectrin is required for early endosome recycling in Drosophila. J. Cell Sci. *119*, 1361–1370. https://doi.org/10.1242/jcs.02839.

Pontes, M.H., and Dale, C. (2006). Culture and manipulation of insect facultative symbionts. Trends Microbiol. *14*, 406–412. https://doi.org/10.1016/j.tim.2006.07.004.

Rämet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., and Ezekowitz, R.A.B. (2002). Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. Coli. Nature *416*, 644–648. https://doi.org/10.1038/nature735.

Ren, C., Webster, P., Finkel, S.E., and Tower, J. (2007). Increased internal and external bacterial load during Drosophila aging without life-span trade-off. Cell Metab. *6*, 144–152. https://doi.org/10.1016/j.cmet.2007.06.006.

Ridley, E.V., Wong, A.C.N., Westmiller, S., and Douglas, A.E. (2012). Impact of the resident microbiota on the nutritional phenotype of drosophila melanogaster. PLoS One *7*. https://doi.org/10.1371/journal.pone.0036765.

Robertson, F.W. (1954). Studies in quantitative inheritance. J. Genet. *52*, 494–520. https://doi.org/10.1007/bf02985075.

Robertson, F.W. (1957). Studies in quantitative inheritance XI. Genetic and environmental correlation between body size and egg production in Drosophila Melanogaster - with one text-figure. J. Genet. *55*, 428–443. https://doi.org/10.1007/BF02984061.

Royet, J., and Dziarski, R. (2007). Peptidoglycan recognition proteins: pleiotropic sensors and effectors of antimicrobial defences. Nat. Rev. Microbiol. *5*, 264–277. https://doi.org/10.1038/nrmicro1620.

Royet, J., Gupta, D., and Dziarski, R. (2011). Peptidoglycan recognition proteins: Modulators of the microbiome and inflammation. Nat. Rev. Immunol. *11*, 837–851. https://doi.org/10.1038/nri3089.

Ryu, J.H., Kim, S.H., Lee, H.Y., Bai, J.Y., Nam, Y.D., Bae, J.W., Lee, D.G., Dong, G.L., Shin, S.C., Seung, C.S., et al. (2008). Innate immune homeostasis by the homeobox gene Caudal and commensal-gut mutualism in Drosophila. Science *319*, 777–782. https://doi.org/10.1126/science.1149357.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods *9*, 671–675. https://doi.org/10.1038/nmeth.2089.

Schwarzer, M., Makki, K., Storelli, G., Machuca-Gayet, I., Srutkova, D., Hermanova, P., Martino, M.E., Balmand, S., Hudcovic, T., Heddi, A., et al. (2016). Lactobacillus plantarum strain maintains growth of infant mice during chronic undernutrition. Science *351*, 854–857. https://doi.org/10.1126/science.aad8588.

Shin, S.C., Kim, S.H., You, H., Kim, B., Kim, A.C., Lee, K.A., Yoon, J.H., Ryu, J.H., and Lee, W.J. (2011). Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science *334*, 670–674. https://doi.org/10.1126/science.1212782.

Sommer, F., and Bäckhed, F. (2013). The gut microbiota-masters of host development and physiology. Nat. Rev. Microbiol. *11*, 227–238. https://doi.org/10.1038/nrmicro2974.

Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J., and Leulier, F. (2011). Lactobacillus plantarum promotes drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. Cell Metab. *14*, 403–414. https://doi.org/10.1016/j.cmet.2011.07.012.

Storelli, G., Strigini, M., Grenier, T., Bozonnet, L., Schwarzer, M., Daniel, C., Matos, R., and Leulier, F. (2018). Drosophila perpetuates nutritional mutualism by promoting the fitness of its intestinal symbiont Lactobacillus plantarum. Cell Metab. *27*, 362–377.e8. https://doi.org/10.1016/j.cmet.2017.11.011.

Takehana, A., Yano, T., Mita, S., Kotani, A., Oshima, Y., and Kurata, S. (2004). Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in Drosophila immunity. EMBO J. *23*, 4690–4700. https://doi.org/10.1038/sj.emboj.7600466.

Troll, J.V., Bent, E.H., Pacquette, N., Wier, A.M., Goldman, W.E., Silverman, N., and McFall-Ngai, M.J. (2010). Taming the symbiont for coexistence: a host PGRP neutralizes a bacterial symbiont toxin. Environ. Microbiol. *12*, 2190–2203. https://doi.org/10.1111/j.1462-2920.2009.02121.x.

Wang, L., Gilbert, R.J.C., Atilano, M.L., Filipe, S.R., Gay, N.J., and Ligoxygakis, P. (2008). Peptidoglycan recognition protein-SD provides versatility of receptor formation in Drosophila immunity. Proc. Natl. Acad. Sci. U S A *105*, 11881–11886. https://doi.org/10.1073/pnas.0710092105.

Wang, M., Liu, L.-H., Wang, S., Li, X., Lu, X., Gupta, D., and Dziarski, R. (2007). Human peptidoglycan recognition proteins require zinc to kill both gram-positive and gram-negative bacteria and are synergistic with antibacterial peptides. J. Immunol. *178*, 3116–3125. https://doi.org/10.4049/jimmunol.178.5.3116.

Werner, T., Borge-Renberg, K., Mellroth, P., Steiner, H., and Hultmark, D. (2003). Functional diversity of the Drosophila PGRP-LC gene cluster in the response to lipopolysaccharide and peptidoglycan. J. Biol. Chem. *278*, 26319–26322. https://doi.org/10.1074/jbc.C300184200.

Wier, A.M., Nyholm, S.V., Mandel, M.J., Massengo-Tiassé, R.P., Schaefer, A.L., Koroleva, I., Splinter-BonDurant, S., Brown, B., Manzella, L., Snir, E., et al. (2010). Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. Proc. Natl. Acad. Sci. U S A *107*, 2259–2264. https://doi.org/10.1073/pnas.0909712107.

Wilson, A.C.C., Ashton, P.D., Calevro, F., Charles, H., Colella, S., Febvay, G., Jander, G., Kushlan, P.F., MacDonald, S.J., Schwartz, J.F., et al. (2010). Genomic insight into the amino acid relations of the pea aphid, Acyrthosiphon pisum, with its symbiotic bacterium Buchnera aphidicola. Insect Mol. Biol. *19*, 249–258. https://doi.org/10.1111/j.1365-2583.2009.00942.x.

Wong, C.N.A., Ng, P., and Douglas, A.E. (2011). Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environ. Microbiol. *13*, 1889–1900. https://doi.org/10.1111/j.1462-2920.2011.02511.x.

Yu, Y., Park, J.W., Kwon, H.M., Hwang, H.O., Jang, I.H., Masuda, A., Kurokawa, K., Nakayama, H., Lee, W.J., Dohmae, N., et al. (2010). Diversity of innate immune recognition mechanism for bacterial polymeric meso-diaminopimelic acid-type peptidoglycan in insects. J. Biol. Chem. *285*, 32937–32945. https://doi.org/10.1074/jbc.M110.144014.

Zaidman-Rémy, A., Hervé, M., Poidevin, M., Pili-Floury, S., Kim, M.S., Blanot, D., Oh, B.H., Ueda, R., Mengin-Lecreulx, D., and Lemaitre, B. (2006). The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection. Immunity *24*, 463–473. https://doi.org/10.1016/j.immuni.2006.02.012.

Zaidman-Rémy, A., Poidevin, M., Hervé, M., Welchman, D.P., Paredes, J.C., Fahlander, C., Steiner, H., Mengin-Lecreulx, D., and Lemaitre, B. (2011). Drosophila immunity: analysis of PGRP-SB1 expression, enzymatic activity and function. PLoS One *6*, e17231. https://doi.org/10.1371/journal.pone.0017231.

Zare, A., Johansson, A.M., Karlsson, E., Delhomme, N., and Stenberg, P. (2018). The gut microbiome participates in transgenerational inheritance of low-temperature responses in Drosophila melanogaster. FEBS Lett. *592*, 4078–4086. https://doi.org/10.1002/1873-3468.13278.

Zhang, G., Wang, W., Deng, A., Sun, Z., Zhang, Y., Liang, Y., Che, Y., and Wen, T. (2012). A Mimicking-of-DNA-methylation-patterns pipeline for overcoming the restriction barrier of bacteria. PLoS Genet. *8*, e1002987. https://doi.org/10.1371/journal.pgen.1002987.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial strains		
L. plantarum: Lp^{NIZO2877}	(Martino et al., 2015a)	LKHZ01000000
L. plantarum: Lp$^{FlyG2.1.8}$	(Martino et al., 2018)	PEBE00000000
L. plantarum: Lp$^{DietG20.2.2}$	(Martino et al., 2018)	PEGW00000000
L. plantarum: Lp$^{\Delta ackA}$	This paper	N/A
L. plantarum: Lp^{WJL}	(Martino et al., 2015b)	LKLZ00000000.
Chemicals, peptides, and recombinant proteins		
Inactivated Dried Yeast	Bio Springer	Springaline BA95/0-PW
Cornmeal	Westhove	Farigel maize H1
Agar	VWR	#20768.361
Methylparaben Sodium Salt	MERCK	106756; CAS: 5026-62-0
Propionic Acid	Sigma-Aldrich	P1386; CAS: 79-09-4
N-Acetyl Glutamine	Sigma-Aldrich	A9125-25G; CAS: 2490-97-3
Man, Rogosa, and Sharpe (MRS) Broth Medium	Condalab	1215
Man, Rogosa, and Sharpe (MRS) Agar Medium	Condalab	1043
Luria Bertani Broth	Condalab	1551
Phosphate buffered saline (PBS)	Sigma-Aldrich	P4417
Glycerol	Sigma-Aldrich	G5516; CAS: 56-81-5
Tetracyclin	Sigma-Aldrich	87128; CAS: 60-54-8
Ampicilin	Sigma-Aldrich	A9393; CAS: 69-53-4
Kanamycin	Sigma-Aldrich	K1377; CAS: 25389-94-0
Erythromycin	Sigma-Aldrich	E1300000; CAS: 114-07-8
RNA later	Invitrogen	AM7021
Critical commercial assays		
RNeasy Mini Kit	Qiagen	74104
Agilent RNA 6000 Nano kit	Agilent	5067-1511
SuperScript™ IV First-Strand Synthesis System	Invitrogen™	18091050
PowerUp™ SYBR™ Green Master Mix	Applied Biosystems™	A25741
Deposited data		
Raw and Analysed Data	This paper	NCBI: Temporary Accession Number SUB10001912
Experimental models: Organisms/strains		
Drosophila melanogaster: *yw* (reference strain for this work)	(Martino et al., 2018)	N/A
Drosophila melanogaster: Oregon R	Department of Biology University of Padova	N/A
Drosophila melanogaster Mex-Gal4	(Phillips and Thomas, 2006)	N/A
Drosophila melanogaster UAS-mCherry-RNAi	BDSC	RRID: BDSC_35785
Drosophila melanogaster: *PGRP-SC*$^{\Delta}$	(Paredes et al., 2011)	N/A
Oligonucleotides		
PGRPSc1-F: AGCTTCCTGGGCAACTACAA	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
PGRPSc1-R: TCGTTCCAGATGTGAGTGCC	This paper	N/A
rp49-F: CCCAAGATCGTGAAGAAGCG	This paper	N/A
rp49-R: TTGTCGATACCCTTGGGCTT	This paper	N/A
AttD-F: AGGAAACCCAAAGAGCGGAG	This paper	N/A
AttD-R: ACTGGACTTACGCGTTGACC	This paper	N/A
Drs_F: CCAAGCTCCGTGAGAACCTT	This paper	N/A
Drs_R: ACAGGTCTCGTTGTCCCAGA	This paper	N/A
oJV1: ATTGCATGCTTATGAGCAGTGAGCT CAAGCTTTCTTTGAA	This paper	N/A
oJV2: AAATTCAGAAGGGATTCTCCACTAGT GGTACCTTAGCTGT	This paper	N/A
oJV3: ACAGCTAAGGTACCACTAGTGGAGA ATCCCTTCTGAATTTAG	This paper	N/A
oJV4: TTCAAAGAAAGCTTGAGCTCACTGCT CATAAGCATGCAAT	This paper	N/A
oJV5: ATGAGTCAATTAAAAACGCGTG	This paper	N/A
oJV6: GACTATTTTTCCTCCATCCAT	This paper	N/A
oJV7: TTTTGCTCACATGTTCTTTC	This paper	N/A
oJV8: CTGCTTTTTGGCTATCAATC	This paper	N/A
oJV9: TCATTTAAATCGCCTTGTCGCA	This paper	N/A
oJV10: CTCACCAACCAAGTCTG	This paper	N/A
oJV11: CATCATGATCGCCTCGCTTTCT	This paper	N/A
Recombinant DNA		
Plasmids used to engineer *L. plantarum* with CRISP-as9, see Table S6	This paper	N/A
Software and algorithms		
ImageJ	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
Rstudio	Rstudio Team, 2015	https://www.rstudio.com/
LyghtCycler 480 Basic Software Version 1.2	Roche Diagnostic	N/A
Automatic Colony Counter Scan® 300 and Software	Vetrotecnica	N/A
Leica application suite (LAS)	Leica	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Maria Elena Martino (mariaelena.martino@unipd.it).

Material availability

Plasmids and bacterial strains generated in this study are available from the lead contact.

Data and code availability

- All data needed to evaluate the conclusions in the article are present in the article or the supplemental information. The Raw data (FASTQ) have been deposited at NCBI under the temporary Accession number SUB10001912.
- No new code was generated during the course of this study.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains and culture conditions

The strains used in the present study are listed in Table S5. All strains were grown in Man, Rogosa, and Sharpe (MRS) broth and agar medium (Condalab, Spain) over night at 37°C without shaking and stored at −80°C in MRS broth containing 20% glycerol.

D. melanogaster strains and maintenance

Drosophila yw flies were used as the reference strain in this work. Other strains used in this work were: Oregon^{R} (flies purchased from Department of Biology, University of Padova, Italy), *PGRP-SC*$^{\Delta}$ KO line (kind gift from Prof. Bruno Lemaitre) described in Paredes et al. (2011), UAS-PGRPSC-RNAi (kind gift from François Leulier) described in Bischoff et al., (2006), and Mex-Gal4, UAS-mCherry-RNAi (kind gift from François Leulier). For experiments, parents were crossed in Drosophila polystyrene vials containing 50 g/L inactivated yeast (rich diet) at 25°C. Progeny were collected at emergence and kept on rich diet at a density of 30 animals/vial for three days, prior to the bacterial mono-association.

Germ-free (GF) stocks were established as described in Storelli et al. (2011). *Drosophila* stocks were kept at 25°C with 12/12 hrs dark/light cycles on a yeast/cornmeal diet containing 50 g/L inactivated yeast (rich diet) as described by Storelli et al. (2011). Poor Yeast Diet (PYD) and PYD + N-acetyl-Glutamine (NAG) were obtained by reducing the amount of yeast extract to 8 g/L and adding 1 g of NAG, respectively, as described in Martino et al., (2018).

Colonization and infection of larvae

GF adults were put overnight in breeding cages to lay eggs on PYD. GF embryos were collected the next morning and seeded by pools of 40 on 55 mm petri dishes containing PYD. Bacterial strains were cultured to stationary phase in MRS broth at 37°C. The embryos were mono-associated with 150 µL (7×10^7 total CFU) of the respective bacteria, or sterile Phosphate buffered saline (PBS, Sigma-Aldrich, USA) for GF condition. Petri dishes were incubated at 25°C until larvae collection.

METHOD DETAILS

Larval size measurements

The size of the larvae was measured for all conditions from day 4 to day 7 of incubation. Larvae ($n \geq 60$) were collected and washed in distiller water, killed with a short heat treatment (5s at 90°C) and transferred on a microscopy slide. Images of the larvae were captured using a digital microimaging Leica DMD108 and larval longitudinal size (length) was measured using ImageJ software (Schneider et al., 2012). The pictures of the larvae were captured with Leica MZ16A stereomicroscope with Leica Application Suite (LAS) microscope software.

Bacterial load quantification

Quantification of larvae-associated bacteria was determined from a pool of 25 larvae (at least three replicates for condition) on day 4. Larvae were surface sterilized in 75% ethanol and placed in 1 mL of PBS. Samples were mechanically crushed, plated on MRS agar and incubated at 37°C for 48 h. For each sample, the remaining fly food was placed in 10 mL of PBS. Samples were plated and incubated as reported above. The CFU count was performed using automatic colony counter Scan® 300 (Vetrotecnica, Italy) and its accompanying software. GF samples were plated on Luria Bertani (LB) agar (Condalab, Spain), and incubated for 72 h at 25°C.

L. plantarum^{NIZO2877} mutant generation

Plasmid generation

Two *E. coli-Lactiplantibacillus* shuttle vectors were used to perform genome editing in *Lp*^{NIZO2877}. The first shuttle vector, pCB578 (Table S6) encodes SpyCas9, a tracrRNA and repeat-spacer-repeat array with a 30-nt spacer targeting the acetate kinase gene (*ackA*) that was used previously in *Lp*^{NIZO2877} (Martino et al., 2018). The second shuttle vector, pCB591, was used to clone a recombineering template to generate a clean deletion of the *ackA* gene in *Lp*^{NIZO2877}. First, pCB591 was amplified with primers oJV1-2, and genomic DNA from *Lp*^{NIZO2877} was amplified with primers oJV3-4 that amplify ackA as

well as 1-kb homology arms flanking the start and stop codons. The PCR fragments were stitched together using the Gibson assembly kit (NEB CN# E2611S) following manufacturer's instructions to yield pJV204. Then, primers oJV5-6 were used to remove *ackA* from pJV204 using the Q5 site-directed mutagenesis kit (NEB CN# E0554S) following the manufacturer's instructions, yielding the final recombineering template pJV218. NEB 5-alpha or 10-beta Competent *E. coli* cells were used for both cloning steps, and primers oJV7-8 were used for screening clones by colony PCR. Correct clones were confirmed by Sanger sequencing.

Transformation of plasmids

Transformation of plasmids into $Lp^{NIZO2877}$ was performed as described previously (Leenay et al., 2019). In short, 1 mL of an overnight culture was used to inoculate 25 mL of fresh MRS supplemented with 2.5% glycine and was grown until OD_{600} reached 0.6–0.8. Then, cells were washed twice with ice-cold 10 mM $MgCl_2$ and twice more with ice-cold SacGly solution. Plasmid DNA (1 μg suspended in water) and 60 μL of electrocompetent cells were added to a pre-cooled 1-mm electroporation cuvette and transformed at the following conditions: 1.8 kV, 200 Ω resistance, and 25 μF capacitance. Following electroporation, cells recovered in MRS broth for 3 hours then were plated on MRS agar containing appropriate antibiotics for 3 days before screening or inoculating colonies. Chloramphenicol and erythromycin concentrations were both 10 μg mL^{-1} in MRS liquid media and agar.

Genome editing

To delete *ackA* from $Lp^{NIZO2877}$, the recombineering template shuttle vector pJV218 was passaged through the methyltransferase-deficient *E. coli* strain EC135 to improve transformation efficiency (Zhang et al., 2012) and then transformed into $Lp^{NIZO2877}$, where transformants were plated on MRS agar plates containing chloramphenicol. Then, the ackA-targeting SpyCas9 shuttle vector pCB578 was passaged through EC135 and transformed into the $Lp^{NIZO2877}$strain harbouring the recombineering template shuttle vector, where transformants were plated on MRS agar containing erythromycin. Surviving colonies were screened for the desired genomic deletion using colony PCR with primers oJV9-10, and the PCR products were subjected to gel electrophoresis and Sanger sequencing with oJV11 to validate the clean deletion of *ackA* (Figure S7). Both plasmids were cured from the mutant *L. plantarum*$^{\Delta ackA}$ strain by cycling between culturing in MRS media and plating on MRS agar, both without antibiotics. After each round of non-selective growth, cultures were plated on MRS agar supplemented with either chloramphenicol or erythromycin. This cycle was repeated until the mutant strain was sensitive to either antibiotic.

RNA extraction, RT-PCR and real-time PCR

Five biological replicates were generated for each condition ($Lp^{NIZO2877}$-, $Lp^{Fly.G2.1.8}$-, and PBS-associated larvae). Twenty-five larvae or thirty-five dissected guts were collected for each sample, transferred in 50 μL of RNA later, flash-frozen in liquid nitrogen and stored at −80°C. RNA was extracted using RNeasy Mini Kit (Qiagen, Germany). Quantity and quality of RNA was assessed on an Agilent 2100 Bioanalyzer using Agilent RNA 6000 Nano kit (Agilent, USA). Extracted RNA was reverse-transcribed to cDNA using SuperScript™ IV First-Strand Synthesis System (Invitrogen™, USA). Real-time PCR amplifications were performed on a LightCycler 480 thermal cycler (Roche Diagnostic, Mannheim, Germany) in a final volume of 10 μL, which included 2.5 μL of cDNA template. The PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, USA) was used together with 0.25 μL of each primer (Table S7). The cycling conditions were as follows: 50°C for 2 min, followed by 2 min at 95°C, and 45 cycles at 95°C for 10 s and 60°C for 1 min. Outputs of real-time amplifications were analysed by means of the LyghtCycler 480 Basic Software Version 1.2 (Roche Diagnostic, Mannheim, Germany). The amount of mRNA detected was normalized to control *rp49* mRNA values. Normalized data were used to quantify the relative levels of a given mRNA according to cycle threshold analysis (ΔC_T).

Library preparation and RNA sequencing

The preparation of mRNA-Seq libraries and their sequencing has been carried out at the EMBL Genomics Core Facilities, Germany. Preparation of barcoded stranded mRNA-Seq libraries was performed with unidirectional deep sequencing of pooled libraries, read-length 80 bases, Illumina NextSeq run (yield ~550 million reads/lane), the pool of 15 libraries in one run.

QUANTIFICATION AND STATISTICAL ANALYSES

Single-end reads have been mapped onto the *D. melanogaster* reference dmel-all-r6.36 with STAR. The read counts were obtained with RSEM and the differential expression analysis has been done on R with DE-seq2 package. For the comparative analysis between gnotobiotic and GF larvae, all samples and the three conditions were added to the model. To compare $Lp^{NIZO2877}$- and $Lp^{Fly.G2.1.8}$-associated larvae samples, a second model was used with only these samples. The GO term analysis was performed using DAVID online tool on the Flybase gene ID (Huang et al., 2009).

Data representation and analysis was performed using Graphpad PRISM 9 software (www.graphpad.com). One–Way ANOVA test was applied to performed statistical analyses between multiple ($n > 2$) conditions for multiple comparisons test ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$). Additional images have been done on R software with ggplot2 package.