

# *Drosophila* Gut Immune Pathway Suppresses Host Development-Promoting Effects of Acetic Acid Bacteria

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The physiology of most organisms, including *Drosophila*, is heavily influenced by their interactions with certain types of commensal bacteria. *Acetobacter* and *Lactobacillus*, two of the most representative *Drosophila* commensal bacteria, have stimulatory effects on host larval development and growth. However, how these effects are related to host immune activity remains largely unknown. Here, we show that the *Drosophila* development-promoting effects of commensal bacteria are suppressed by host immune activity. Mono-association of germ-free *Drosophila* larvae with *Acetobacter pomorum* stimulated larval development, which was accelerated when host immune deficiency (IMD) pathway genes were mutated. This phenomenon was not observed in the case of mono-association with *Lactobacillus plantarum*. Moreover, the mutation of Toll pathway, which constitutes the other branch of the *Drosophila* immune pathway, did not accelerate *A. pomorum*-stimulated larval development. The mechanism of action of the IMD pathway-dependent effects of *A. pomorum* did not appear to involve previously known host mechanisms and bacterial metabolites such as gut peptidase expression, acetic acid, and thiamine, but appeared to involve larval serum proteins. These findings may shed light on the interaction between the beneficial effects of commensal bacteria and host immune activity.

**Keywords:** *Acetobacter*, *Drosophila*, host-microbe interaction, IMD immune pathway, *Lactobacillus*, Toll immune pathway

## INTRODUCTION

An organism establishes complex interactions with its microbiome, and such interactions hugely impact various aspects of its physiology. Commensal bacteria and their host can provide reciprocal functional benefits through mutualistic interactions. However, the association of pathogenic bacteria with a host could be beneficial for the bacteria but deleterious for the host. Despite the increased interest in the functional interactions between commensal bacteria and their hosts, a comprehensive view of this interaction is yet to be established due to its high level of complexity. To understand the basic principles governing complex host-microbe interactions, the development of a relatively simple model system is required. *Drosophila* has recently emerged as an important model organism to study such interactions (Douglas, 2018; 2019; Matos and Leulier, 2014; Wong et al., 2016). The powerful genetic tools available for studies in *Drosophila*, coupled with the simplicity of the cultivation of germ-free (GF) *Drosophila* for manipulating its commensal bacterial species, makes *Drosophila* an ideal host model to study the molecular mechanisms underlying bacteria-mediated physiological benefits.

*Drosophila* is associated with bacterial communities of relatively low complexity, consisting of a handful of species dominated by the members of the *Acetobacteraceae* and *Lactobacillaceae* families (Douglas, 2018; 2019; Han et al., 2017). It is known that certain bacterial species from the *Lac-*

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*tobacillus* and *Acetobacter* genera promote fly larval development and growth especially upon undernutrition. In a GF condition, an undernourished fly larva shows stunted body growth and delayed development, which could be rescued by mono-association with specific commensal bacterial species, such as *Lactobacillus plantarum* (Lp) and *Acetobacter pomorum* (Ap) (Erkosar et al., 2015; Lee et al., 2022; Matos et al., 2017; Shin et al., 2011; Storelli et al., 2011). *L. plantarum* exerts its beneficial effect on larval development through the host nutrient-sensing system, which depends on TORC1 activity in the adipose organ of the *Drosophila* and, subsequently, modulates the hormones regulating growth and maturation (Storelli et al., 2011). Other studies also showed that *L. plantarum* can enhance the efficiency of host digestion by promoting enzyme activities and, hence, stimulating sufficient extraction of dietary nutrients, which may support the efficient growth of larva supplied with a poor-protein diet (Erkosar et al., 2015; Lee et al., 2020; Matos et al., 2017). Likewise, mono-association of a GF fly with *A. pomorum* significantly rescues stunted larval development and the small size of the adult body by enhancing systemic IIS (insulin/IGF-1 [insulin-like growth factor 1] signaling) (Shin et al., 2011). Interestingly, mutant *A. pomorum* bacteria lacking quinone-dependent alcohol dehydrogenase (PQQ-ADH) activity fail to rescue the phenotypes of GF fly, which are further rescued by the addition of acetic acid in the diet (Shin et al., 2011). The requirement of acetic acid for normal fly development and survival has been further supported by the finding that the lethality of *Drosophila* infected with *Vibrio cholera* depends on the transition of the bacteria from excretion to assimilation of gut environmental acetic acid (Hang et al., 2014). Moreover, a recent study showed that thiamine (vitamin B<sub>1</sub>) provided by *Acetobacter* among the fly microbiota supports fly development (Sannino et al., 2018), and riboflavin (vitamin B<sub>2</sub>) was found to be a microbial factor that contributes to *Acetobacter*-mediated transgenerational effects of an antibiotic on fly development (Fridmann-Sirkis et al., 2014). These findings indicate that the metabolic products of *Acetobacter* are the critical factors that confer beneficial effects of commensal bacteria on fly larval development.

Previous studies have unveiled the relationship between the innate immune activities of the *Drosophila* gut environment and commensal bacteria. The gut microbiota has a significant influence on the maintenance of host immune homeostasis (Bischoff et al., 2006; Bosco-Drayon et al., 2012; Buchon et al., 2009; Ha et al., 2009; Kim and Lee, 2021; Lhocine et al., 2008; Paredes et al., 2011; Ryu et al., 2008). Frequent perturbation of innate immune homeostasis seen in old flies is accompanied by dysbiotic microbiota associated with excessive proliferation of intestinal stem cells and intestinal dysplasia (Broderick and Lemaitre, 2012; Buchon et al., 2009; Guo et al., 2014). A recent study has reported the requirement of gut immune activity and commensal bacteria-produced acetic acid in the maintenance of fly metabolic homeostasis (Kamareddine et al., 2018). Despite these studies demonstrating the interactions between commensal bacteria and the host immune system, it is still unknown whether commensal bacteria-associated immune activity is linked to the bacterial effects on host larval development, and the un-

derlying mechanism remains elusive.

In this study, we showed that host immune activity has suppressive effects on host larval development stimulated by specific commensal bacterial species. Mutation of the host immune deficiency (IMD) pathway accelerated *A. pomorum*-stimulated larval development. This phenomenon is immune pathway- and bacterial species-specific, since it was not observed when the Toll pathway was mutated or when *Drosophila* was mono-associated with *L. plantarum* instead of with *A. pomorum*. Further, we provide evidence that this effect of *A. pomorum* is neither mediated by acetic acid and thiamine, the metabolites of *A. pomorum* known to enhance larval development, nor by increasing peptidase expression in the gut, but is mediated by gut expression of *larval serum protein* (*Lsp*) in response to Ap mono-association.

## MATERIALS AND METHODS

### Fly rearing

The *w<sup>1118</sup>* (BL 3605), *rel<sup>E20</sup>* (BL 55714), *imd<sup>1</sup>* (BL 55711), *Myo1A Gal4* (BL 67057), *UAS-Lsp1 $\alpha$*  RNAi (BL 56039), *UAS-Lsp1 $\beta$*  RNAi (BL 27042), *UAS-Lsp1 $\gamma$*  RNAi (BL 55389), and *UAS-Lsp2* RNAi (BL 57505) flies were obtained from Bloomington Stock Center (USA). The *Canton S*, *yw*, and *spz<sup>m7</sup>* flies were described elsewhere (Tzou et al., 2002). *UAS-imd* RNAi (VDRC 9253) and *UAS-rel* RNAi (VDRC 49413) flies were obtained from the Vienna RNAi Library Center. *PGRP-LC<sup>E12</sup>*, *PGRP-LC<sup>E12</sup>*, and *dif<sup>1</sup>* flies were generous gifts from Dr. Won-Jae Lee (Seoul National University, Korea). All flies used in this study were maintained at 25°C under 50% humidity on low-yeast fly food (86.2 g/L glucose, 40.8 g/L cornmeal, 15.6 g/L dried yeast, and 9.3 g/L agar) and a 12-h light/dark cycle.

### Bacterial culture

*A. pomorum<sup>DM001</sup>*, *A. pomorum<sup>P3G5</sup>*, and *L. plantarum<sup>WJL</sup>* were obtained from Dr. Won-Jae Lee (Seoul National University) and maintained in our lab. The bacteria used in this study were cultured in Man, Rogosa, and Sharpe (MRS) broth medium. *A. pomorum<sup>DM001</sup>* and *A. pomorum<sup>P3G5</sup>* were grown for 36 h at 30°C with shaking; *L. plantarum<sup>WJL</sup>* was incubated overnight at 37°C without shaking.

### Mono-association of GF flies with bacteria

Embryos laid for approximately 6 h on apple juice agar plates by young female flies were collected. To prepare GF embryos, collected embryos were placed in a 50% bleach solution for 90 s, rinsed twice with 70% ethanol, and then washed with sterile distilled water three times. GF embryos were transferred to vials supplemented with sterilized fly food using a thin brush and maintained at 25°C. The axenic state of the flies and food was tested by culturing homogenates on the nutrient medium. Bacterial culture (150  $\mu$ l, OD<sub>600</sub> = 1) was added directly onto the GF embryos and the sterilized fly food. Each fly strain monoassociated with bacteria was homogenized and cultured on nutrient agar plates in order to ensure gnotobiotic conditions.

### Pupariation time measurement

To determine pupariation time, the number of pupae that

passed the late 3rd instar larval stage was counted every 12 h until all larvae pupariated. By measuring the time when 50% of total pupal number was formed, the actual median pupariation times represented as days were determined. The actual median pupariation times were normalized to that of GF larva to yield relative pupariation times. Approximately 30 larvae were reared in each vial to avoid overcrowding. Each graph represents the mean of at least three biological replicates. The graphs of actual median pupariation times are provided in supplementary figures.

### RNA preparation and reverse transcription followed by quantitative PCR

Larval organ samples (gut, fat body, and muscle) were quickly transferred into TRIzol solution (Invitrogen, USA) and ground for RNA analysis. RNA was extracted following the manufacturer's instructions and used for cDNA synthesis using RevertAid Reverse Transcriptase (Thermo Fisher Scientific, USA). Quantitative PCR (qPCR) was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) and THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan). The mRNA levels of interest were calculated as a relative fold-change normalized to *Rp49* mRNA levels and estimated by the comparative Ct (cycle threshold) method. The following PCR primers were used: *diptericin*, 5'-TCC GAT GCC CGA CGA CAT GA-3' and 5'-TGG CGT CCA TTG TCG CTG GT-3'; *jon66Cii*, 5'-AAA CTG ACC CCG GTC CAC-3' and 5'-CCT CCT CAG CCG GAT AGC-3'; *jon99Ci*, 5'-TCC ATA ATC GGA CAC ACT TGG-3' and 5'-CAG TGA AGC CTC ATC AGC AC-3'; *CG18179*, 5'-ACC GAT GGC AAA TCC TCT T-3' and 5'-GCG TTG TCA TGG GTA ACG A-3'; *Lsp1 $\alpha$* , 5'-CAC ACG GAC ATG GAC AGA CA-3' and 5'-GCC AGT TGC TTT CCT GGT TG-3'; *Lsp1 $\beta$* , 5'-CCG AGG CCA TCG AGT TCA TT-3' and 5'-GGC GAG CAT GTA CCA GAA CT-3'; *Lsp1 $\gamma$* , 5'-CTT CGG CTA TCC CTT CGA CC-3' and 5'-TGC CGA ACT GGG TGT ACT TC-3'; *Lsp2*, 5'-AGT CCA CCT TGT GAC CAT CG-3' and 5'-ATT CGT CTG CTA AAA ACT GTA GGC-3'; *Rp49*, 5'-AGG GTA TCG ACA ACA GAG TG-3' and 5'-CAC CAG GAA CTT CTT GAA TC-3'.

### Bacterial load analysis

The bacterial loads of larval gut at 2nd and 3rd instars were quantified by plating a serial dilution of lysates obtained from at least five larval midgut samples on MRS agar plates. The larval midgut was isolated from whole dissected guts, from which the foregut, the hindgut, and the malpighian tubules were carefully removed. Plates were incubated overnight at 30°C or 37°C as specified in the bacterial culture section of Materials and methods. The bacterial load of each strain was quantified by counting the number of colony-forming units (CFUs) on plates.

### Thiamine treatment

To test the effect of thiamine treatment on larval development, we prepared a thiamine solution by mixing thiamine hydrochloride (T4625; Sigma, USA) with sterile distilled water. Four different thiamine solutions containing 0.00, 0.04, 0.2, or 1.0  $\mu\text{g/ml}$  were prepared and 100  $\mu\text{l}$  of each was added to GF embryos grown on sterilized food vials.

### RNA sequencing

RNA was extracted from 15 dissected midgut of four separate larval samples: GF and Ap(DM001) mono-associated larva both in wild-type and *ref<sup>E20</sup>* fly using TRIzol solution (Invitrogen). The libraries of cDNA were generated using an Illumina TruSeq Stranded mRNA LT Sample Prep kit according to the manufacturer's protocol. The Illumina sequencer generates raw images utilizing sequencing control software for system control and base calling through an integrated primary analysis software called RTA (real-time analysis). The BCL (binary base call) files were converted into FASTQ format by utilizing the Illumina package bcl2fastq. RNA-sequencing was performed by Macrogen (Korea). The transcript expression levels were expressed in fragments per kilobase of transcript per million fragments mapped (FPKM).

### Identification of differentially expressed genes

We defined genes with FPKM values of over 1.0 as expressed genes in wild-type and *ref<sup>E20</sup>* fly larval gut following Ap(DM001) mono-association. After converting the FPKM values of each sample to  $\log_2(\text{FPKM} + 1)$ , we identified DEGs (differentially expressed genes) between GF and Ap(DM001) mono-associated samples of wild-type and *ref<sup>E20</sup>* fly as genes with a fold change of over 1.5. Finally, a list of genes upregulated to a greater extent by Ap(DM001) in *ref<sup>E20</sup>* fly compared with wild-type fly was displayed on a heatmap using the Prism software (ver. 8.0.1; GraphPad Software, USA). To assess enriched biological themes among candidate genes, the three Gene Ontology (GO) terms "molecular function", "cellular component", and "biological process" were analyzed using the database for annotation, visualization, and integrated discovery (DAVID) software.

### Quantification and statistical analysis

The Prism software (ver. 8.0.1) was used for all statistical analysis. All error bars indicate SEM. Comparisons of multiple samples were performed by ordinary one-way ANOVA, followed by post hoc Dunnett's test to calculate the statistical significance of the differences compared with the mean of the corresponding control group. For all results, asterisks are used to indicate statistical significance as follows: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , and the differences that were not statistically significant are marked by "n.s.". The number of biological replicates, actual  $P$  values, and  $F$  values for ANOVA are noted in figure legends.

## RESULTS

### Mutation of IMD immune pathway genes accelerates larval development stimulated by *A. pomorum*, but not by *L. plantarum*, mono-association

The *Drosophila* immune system is composed of the immune deficiency (IMD) pathway and the Toll pathway as well as hemocyte activity (Buchon et al., 2014; Koranteng et al., 2022). First, we investigated the effects of IMD immune activity on larval development stimulated by Ap mono-association. In wild-type flies, mono-association of GF larva with wild-type *A. pomorum*<sup>DM001</sup> [Ap(DM001)] stimulated larval development by 23% compared with GF larva; this expected

phenomenon was observed by monitoring the pupariation time (Figs. 1A and 1D, Supplementary Fig. S1A). The mutant Ap [Ap(P3G5)], which lacks PQQ-ADH activity, did not significantly stimulate larval development, serving as a negative control for our experimental condition (see below). In flies with either mutated *imd* or *rel*, which are the genes encoding components of the IMD immune pathway (Buchon et al., 2014), mono-association of GF larva with Ap(DM001) stimulated larval development by 30% or 40%, respectively, compared with GF larva; these were significantly greater than the 23% stimulation observed in wild-type flies (Figs. 1B-1D, Supplementary Figs. S1B and S1C). Consistently, the stronger effects of Ap in flies with IMD pathway mutations were also observed in flies with mutations in either *PGRP-LE* or *PGRP-LC*, the genes encoding receptors for the systemic activation of the IMD pathway (Supplementary Figs. S2A-S2C).

*L. plantarum*<sup>WJL</sup> [Lp(WJL)] is known to promote growth of infant mice as well as that of fly larvae (Matos and Leulier, 2014; Matos et al., 2017; Schwarzer et al., 2016; Storelli et al., 2011). As reported previously, mono-association of wild-type GF larva with Lp(WJL) stimulated larval development by 31% (Fig. 1A, Supplementary Fig. S1A). However, in contrast with Ap mono-association, Lp(WJL) mono-association of neither *imd* nor *rel* mutant GF larva stimulated development to an extent greater than that observed in wild-type GF larva. Specifically, in flies with either mutated *imd* or *rel*, mono-association of GF larva with Lp(WJL) induced a stimulation of larval development by 29% or 32%, respectively, which were not significantly greater than the 31% difference observed in wild-type flies (Figs. 1B-1D, Supplementary Figs. S1B and S1C).

The comparison of pupariation times between Ap mono-association and Lp mono-association groups showed that Ap mono-association caused pupariation in IMD pathway mutants earlier than that in the wild-type larva, contrasting with the Lp(WJL) mono-associated flies, which showed comparable pupariation timing in the mutant and the wild-type larva (Figs. 1D, Supplementary Fig. S2D). Taken together, these observations indicate that the activity of the IMD immune pathway does not suppress the stimulatory effects of Lp on fly larval development, but specifically suppresses the stimulatory effects of Ap on fly larval development.

#### Knockdown of IMD immune pathway genes in gut enterocytes accelerates larval development stimulated by *A. pomorum*, but not by *L. plantarum*, mono-association

To further confirm that the effects of the IMD pathway mutations on Ap-specific developmental stimulation, we examined the effects of knockdown (KD) of IMD pathway genes using RNA interference (RNAi). In control flies (Myo1A/+), mono-association of GF larva with wild-type Ap(DM001) stimulated larval development by 26% compared with GF larva (Figs. 2A and 2D, Supplementary Fig. S3A). In flies deficient in either *imd* or *rel* in gut enterocytes (gene knockdown in gut enterocytes was performed using *Myo1A gal4*; see below), mono-association of GF larva with Ap(DM001) stimulated larval development by 36% or 37%, respectively, compared with GF larva; these changes were significantly greater than the 26% difference observed in control flies (Figs. 2B-

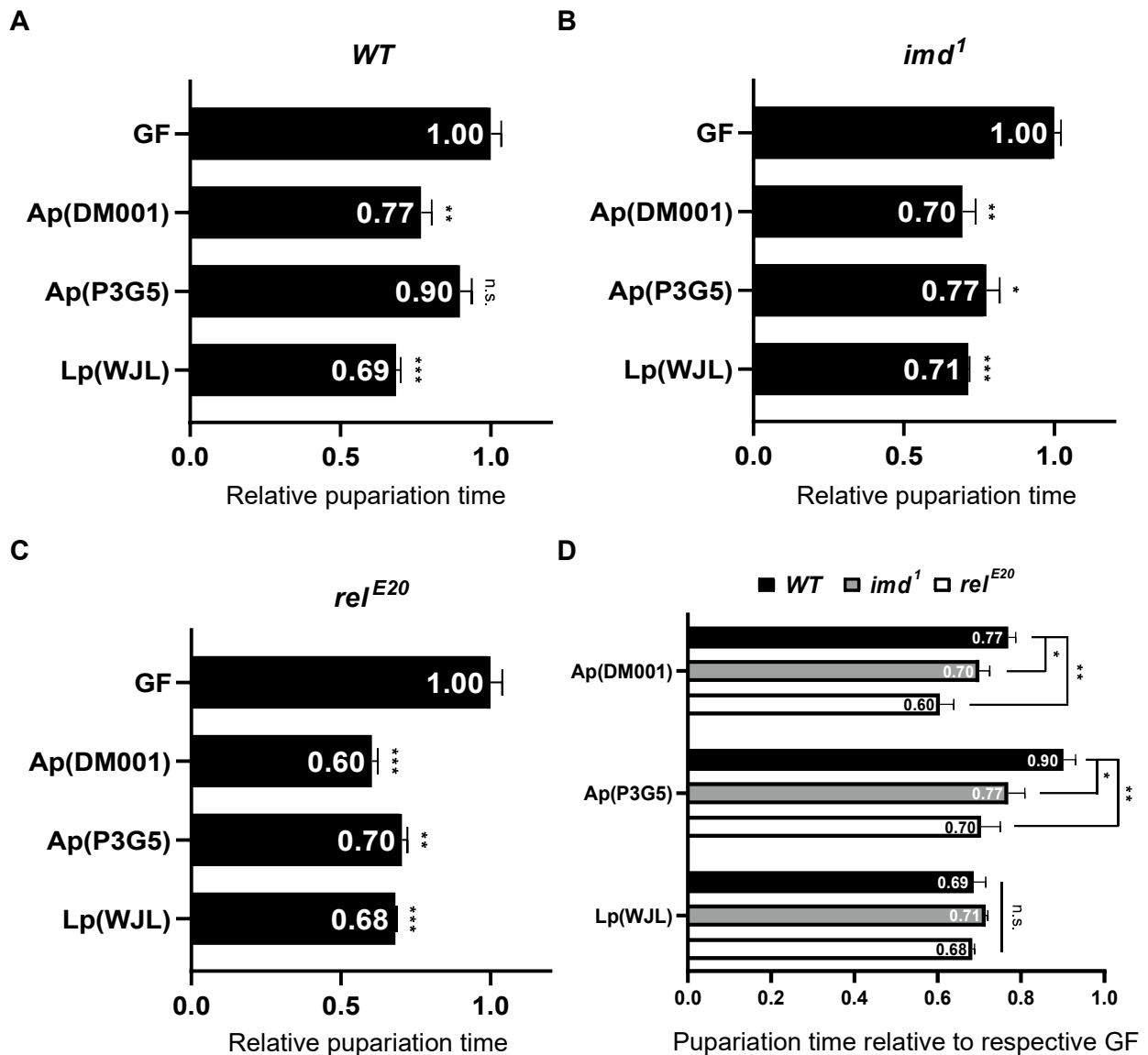
2D, Supplementary Figs. S3B and S3C). Mono-association of control GF larva with Lp(WJL) stimulated larval development by 17% compared with GF (Fig. 2A, Supplementary Fig. S2A). However, in contrast to Ap mono-association, Lp(WJL) mono-association of neither *imd*-KD nor *rel*-KD GF larva stimulated development further than that observed in control GF larva: In *imd*-KD and *rel*-KD flies, mono-association of GF larva with Lp(WJL) stimulated larval development by 21% or 24% compared with GF larva, respectively, which were not significantly greater than the 17% difference observed in wild-type flies (Figs. 2B-2D, Supplementary Figs. S2B and S2C).

Comparison of pupariation times between Ap mono-association and Lp mono-association groups showed that Ap mono-association caused pupariation in the larva with RNAi of IMD pathway genes earlier than it did in the control larva, contrasting with the Lp(WJL) mono-associated flies, which showed comparable pupariation timing in the IMD pathway-deficient (RNAi) and the control larva (Fig. 2D). Together with the results shown in Fig. 1, the data suggest that IMD immune activity in larval gut enterocyte represses the development-promoting effect of Ap, but not that of Lp.

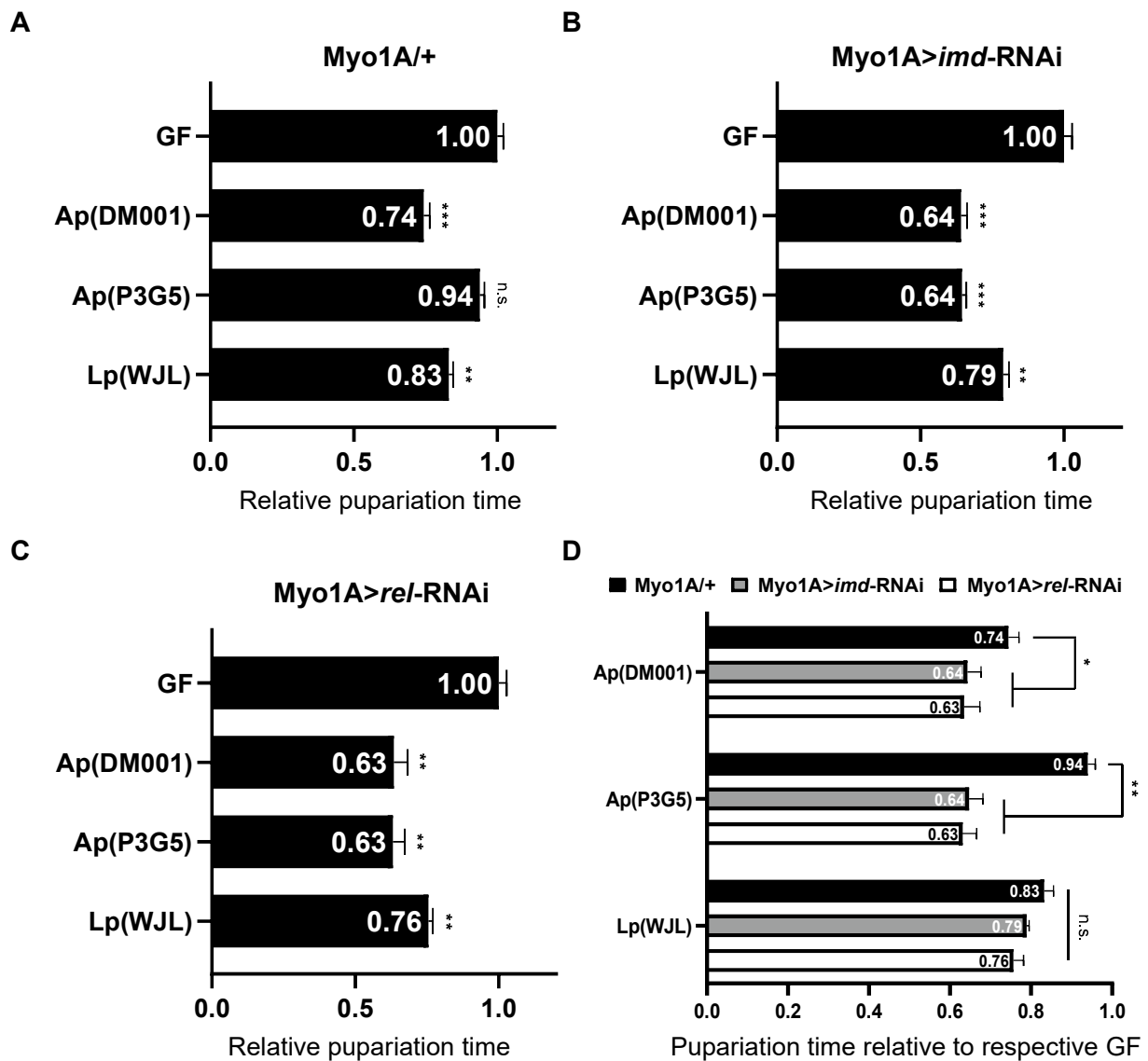
#### Mutation of Toll immune pathway does not accelerate larval development stimulated by *A. pomorum* or *L. plantarum* mono-association

The Toll immune pathway is the other branch of fly innate immunity known to be activated mainly by infection of gram-positive bacteria or fungi. Upon infection, the activation of a protease cascade converts the Toll receptor ligand encoded by *spatzle* (*spz*) into the active form, which stimulates the Toll immune pathway (Buchon et al., 2014). Thus, using a *spz*-mutant host, we tested whether the Toll immune pathway functions in the same manner as the IMD immune pathway in suppressing development-promoting effects of commensal bacteria. As shown in Fig. 1A, mono-association of GF larva with wild-type Ap(DM001) stimulated larval development, while that with Ap(P3G5) did not, serving as a negative control for the experimental setting (Fig. 3A, Supplementary Fig. S4A). In the *spz* mutant flies, mono-association of GF larva with Ap(DM001) stimulated larval development by 18%, the extent of which was lower than that observed in wild-type flies (26%). Mono-association with Ap(P3G5) failed to stimulate the development of *spz* mutant GF larva as well as that of wild-type GF larva (Fig. 3B, Supplementary Fig. S4B). To further confirm this finding, we additionally performed experiments with flies deficient in *dorsal-related immunity factor* (*dif*), another Toll pathway mutant. As observed in *spz* mutant flies, mono-association of *dif* GF larva with Ap stimulated larval development to a similar extent as that observed in wild-type GF larva (Fig. 3C, Supplementary Fig. S4C). These results indicate that in contrast to IMD, the Toll immune pathway does not suppress the stimulatory effects of Ap on fly larval development. In concurrence with the observation that IMD pathway deficiency did not accelerate the larval development stimulated by Lp(WJL), the mutation of neither *spz* nor *dif* accelerated larval development stimulated by Lp(WJL) (Fig. 3, Supplementary Fig. S3). We noted that the larval development stimulated by mono-association

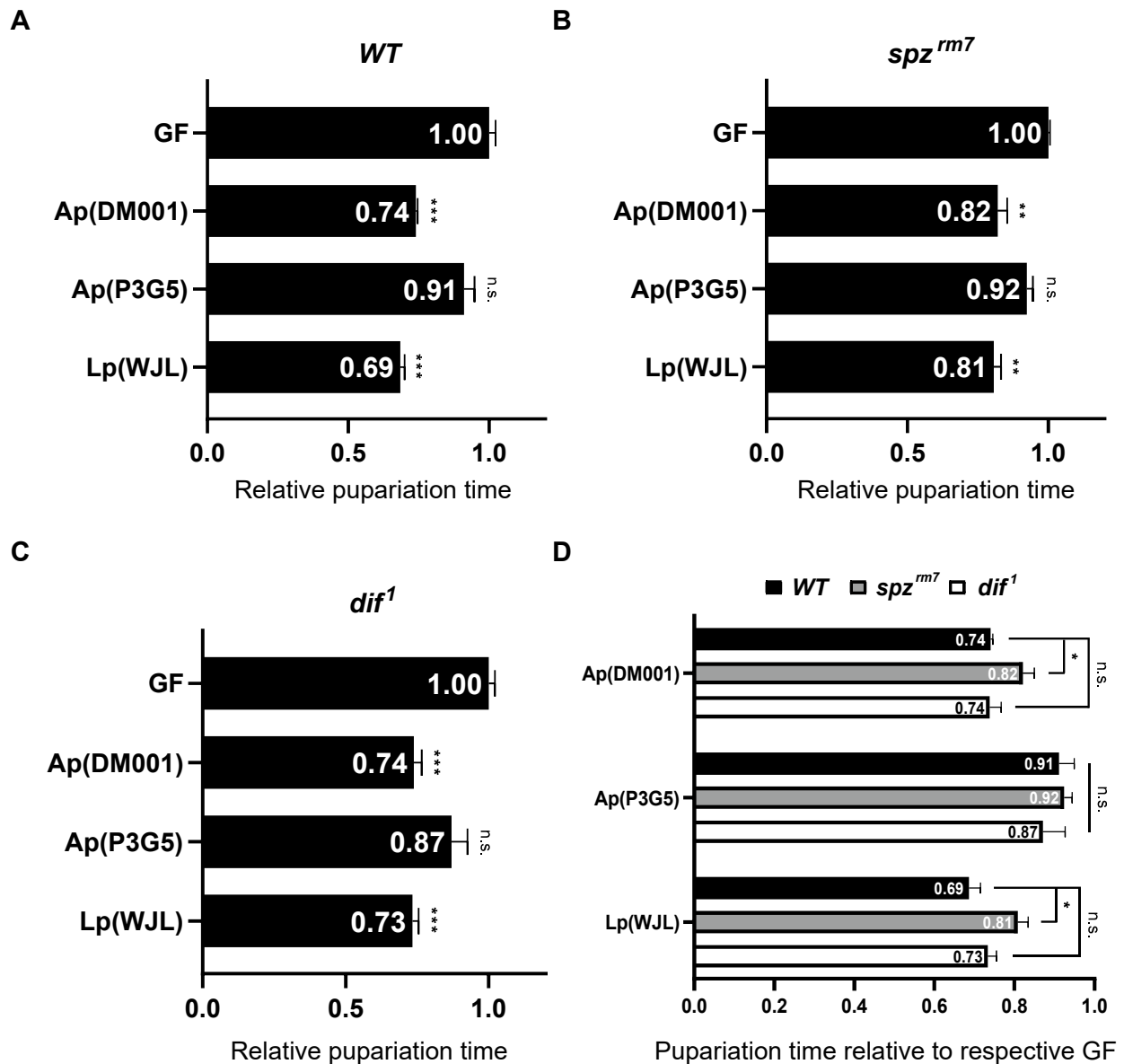




**Fig. 1. Mutation of IMD immune pathway genes accelerates Ap-induced pupariation.** (A) Mono-association of GF wild-type (WT) larva with wild-type Ap [Ap(DM001)] advanced the pupariation timing. Mono-association of GF larva with mutant Ap [Ap(P3G5)] did not significantly advance pupariation timing compared to GF larva. Mono-association of GF wild-type larva with wild-type Lp [Lp(WJL)] advanced pupariation timing. The *P* values for the comparisons with the control group were as follows: Ap(DM001), 0.0013; Ap(P3G5), 0.1443; Lp(WJL), 0.0002. One-way ANOVA ( $F_{[3,20]} = 20.94$ ;  $P = 0.0004$ ), Dunnett's multiple comparisons test; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . (B and C) Mono-association of GF larva of either *imd* (B) or *rel* (C) mutants with Ap(DM001) advanced pupariation timing. Mono-association of GF larva with Ap(P3G5) advanced pupariation timing similarly to Ap(DM001). Mono-association of GF larva with Lp(WJL) advanced pupariation timing similarly as seen in wild-type larva (A). In panel B, the *P* values for the comparisons with the control group were as follows: Ap(DM001), 0.0041; Ap(P3G5), 0.0132; Lp(WJL), 1.51E-05. One-way ANOVA ( $F_{[3,20]} = 18.16$ ;  $P = 0.0006$ ), Dunnett's multiple comparisons test; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . In panel C, the *P* values for the comparisons with the control group were as follows: Ap(DM001), 0.0009; Ap(P3G5), 0.0032; Lp(WJL), 3.48E-06. The *F* value was 47.14. One-way ANOVA ( $F_{[3,20]} = 47.14$ ;  $P < 0.0001$ ), Dunnett's multiple comparisons test; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . (D) Comparison of pupariation timing with respect to the indicated combinations of bacteria and host conditions. Ap(DM001) and Ap(P3G5) advanced the pupariation timing of the IMD pathway-mutant larva more strongly than that they advanced that of the wild-type larva. Lp(WJL) did not differentially advance pupariation times of wild-type and IMD pathway mutant larva. The *P* values for the comparisons with the control (WT) group were as follows: Ap(DM001) in *imd*, 0.0485; Ap(DM001) in *rel*, 0.0201; Ap(P3G5) in *imd*, 0.0491; Ap(P3G5) in *rel*, 0.0147; Lp(WJL) in *imd*, 0.3638; Lp(WJL) in *rel*, 0.2333. One-way ANOVA, Dunnett's multiple comparisons test; \* $P < 0.05$ ; \*\* $P < 0.01$ . Pupariation times were normalized to that of GF larva. The results were obtained from six independent observations, each with 30 larvae. n.s., not statistically significant. Error bars indicate SEM. See also [Supplementary Fig. S1](#). IMD, immune deficiency; Ap, *Acetobacter pomorum*; GF, germ-free; Lp, *Lactobacillus plantarum*.



**Fig. 2. Gut enterocyte-specific knockdown of IMD immune pathway genes accelerates Ap-induced pupariation.** (A) Mono-association of GF control larva (*Myo1A*<sup>+/+</sup>) with wild-type (WT) Ap [Ap(DM001)] advanced the pupariation timing. Mono-association of GF larva with mutant Ap [Ap(P3G5)] did not significantly advance pupariation timing compared to GF larva. Mono-association of GF wild-type larva with wild-type Lp [Lp(WJL)] advanced pupariation timing. The *P* values for the comparisons with the control group were as follows: Ap(DM001), 0.0009; Ap(P3G5), 0.0863; Lp(WJL), 0.0021. One-way ANOVA ( $F_{[3,20]} = 37.78$ ;  $P < 0.0001$ ), Dunnett's multiple comparisons test; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . (B and C) Mono-association of GF larva deficient in either *imd* (B) or *rel* (C) with Ap(DM001) advanced pupariation timing. Mono-association of GF larva with Ap(P3G5) advanced pupariation timing similarly to Ap(DM001). Mono-association of GF larva with Lp(WJL) advanced pupariation timing similarly as seen in control larva (A). In panel B, the *P* values for the comparisons with the control group were as follows: Ap(DM001), 0.0005; Ap(P3G5), 0.0004; Lp(WJL), 0.0003. One-way ANOVA ( $F_{[3,20]} = 57.54$ ;  $P < 0.0001$ ), Dunnett's multiple comparisons test; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . In panel C, the *P* values for the comparisons with the control group were as follows: Ap(DM001), 0.0023; Ap(P3G5), 0.0011; Lp(WJL), 0.0010. One-way ANOVA ( $F_{[3,20]} = 25.77$ ,  $P = 0.0002$ ), Dunnett's multiple comparisons test; \*\* $P < 0.01$ . (D) Comparison of pupariation timing with respect to the combination of the different bacteria and host conditions. Ap(DM001) and Ap(P3G5) advanced the pupariation timing of the IMD pathway-silenced larva more strongly than they advanced that of the control larva. Lp(WJL) did not differentially advance pupariation times of control or IMD pathway-silenced larva. The *P* values for the comparisons with the control (WT) group were as follows: Ap(DM001) in *Myo1A>imd-RNAi*, 0.0150; Ap(DM001) in *Myo1A>rel-RNAi*, 0.0189; Ap(P3G5) in *Myo1A>imd-RNAi*, 0.0014; Ap(P3G5) in *Myo1A>rel-RNAi*, 0.0024; Lp(WJL) in *Myo1A>imd-RNAi*, 0.0558; Lp(WJL) in *Myo1A>rel-RNAi*, 0.0596. One-way ANOVA, Dunnett's multiple comparisons test; \* $P < 0.05$ ; \*\* $P < 0.01$ . Pupariation times were normalized to that of GF larva. The results were obtained from six independent observations, each with 30 larvae. n.s., not statistically significant. Error bars indicate SEM. See also [Supplementary Fig. S3](#). IMD, immune deficiency; Ap, *Acetobacter pomorum*; GF, germ-free; Lp, *Lactobacillus plantarum*.



**Fig. 3. Mutation of Toll immune pathway genes fails to accelerate Ap-induced and Lp-induced pupariation.** (A) Mono-association of GF wild-type (WT) larva with Ap(DM001) or Lp(WJL) advanced pupariation timing, while mono-association of GF larva with Ap(P3G5) did not significantly advance pupariation timing compared to GF larva. The *P* values for the comparisons with the control group were as follows: Ap(DM001), 3.36E-06; Ap(P3G5), 0.0581; Lp(WJL), 0.0002. One-way ANOVA ( $F_{[3,20]} = 318.3$ ;  $P < 0.0001$ ), Dunnett's multiple comparisons test;  $***P < 0.001$ . (B and C) Mono-association of GF larva of either *spz* (B) or *dif* (C) mutants with Ap strains advanced the pupariation timing in a manner similar to that observed in wild-type larva (A). Mono-association of GF larva of either *spz* (B) or *dif* (C) mutants with Lp(WJL) slightly delayed pupariation timing compared to wild-type larva. In panel B, the *P* values for the comparisons with the control group were as follows: Ap(DM001), 0.0061; Ap(P3G5), 0.0568; Lp(WJL), 0.0082. One-way ANOVA ( $F_{[3,20]} = 11.58$ ;  $P < 0.0028$ ), Dunnett's multiple comparisons test;  $**P < 0.01$ . In panel C, the *P* values for the comparisons with the control group were as follows: Ap(DM001), 0.0011; Ap(P3G5), 0.0621; Lp(WJL), 0.0033. One-way ANOVA ( $F_{[3,20]} = 23.70$ ;  $P < 0.0002$ ), Dunnett's multiple comparisons test;  $***P < 0.001$ . (D) The Ap strains and Lp(WJL) did not more strongly advanced the pupariation time of Toll pathway mutant larva such as *spz* or *dif* compared with wild-type larva. The *P* values for the comparisons with the control (WT) group were as follows: Ap(DM001) in *spz*, 0.0301; Ap(DM001) in *dif*, 0.3836; Ap(P3G5) in *spz*, 0.3119; Ap(P3G5) in *dif*, 0.2103; Lp(WJL) in *spz*, 0.0311; Lp(WJL) in *dif*, 0.1970. One-way ANOVA, Dunnett's multiple comparisons test;  $*P < 0.05$ . Pupariation times were normalized to that of GF. The results were obtained from six independent observations, each with 30 larvae. n.s., not statistically significant. Error bars indicate SEM. See also [Supplementary Fig. S4](#). Ap, *Acetobacter pomorum*; Lp, *Lactobacillus plantarum*; GF, germ-free.

of either Ap(DM001) or Lp(WJL) was suppressed by *spz* mutation, suggesting that the commensal bacteria may negatively impact the fitness of the host deficient in Toll immune pathway, thereby slowing larval development (Figs. 3B and 3D).

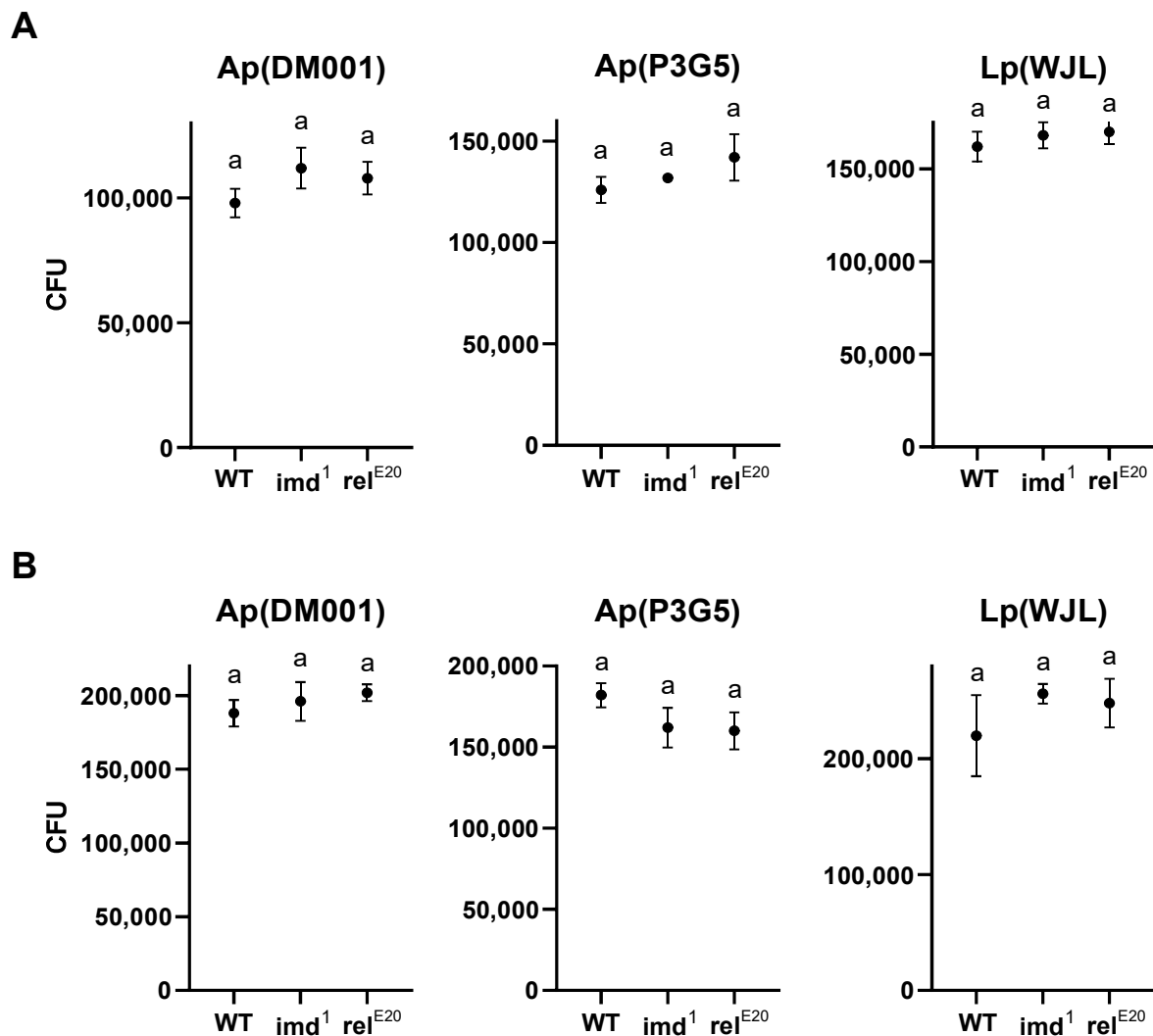
#### Differential responses of immune pathway mutations to Ap and Lp may not be specific to the genetic background of wild-type flies

To exclude the possibility that differential responses of immune pathway mutations to Ap and Lp might arise from different wild-type genetic backgrounds in addition to the immune pathway mutations, we tested several strains of wild-type flies: *Canton S*, *yw*, *w<sup>1118</sup>*. We found that mono-association of Ap or Lp similarly influenced the rate of larval development regardless of the strains of wild-type flies (Sup-

plementary Fig. S5), indicating that the differential effects of commensal bacteria on larval development were not specific to the wild-type genetic background of the flies.

#### The bacterial loads in the larval gut are similar in wild-type and mutant hosts

It could be hypothesized that immune activity compromised by mutation of the IMD pathway allows the proliferation of gut bacteria having development-promoting effects, thereby accelerating larval development. To test this idea, bacterial load in the larval gut during larval development were measured in wild-type and immune pathway-mutant hosts. The CFUs, reflecting bacterial load, in the guts of 2nd (L2) and 3rd (L3) instar larvae showed that mutations in IMD pathway genes did not significantly increase the abundance of Ap or Lp (Figs. 4A and 4B). Likewise, bacterial load in the gut was



**Fig. 4. Similar bacterial loads in the guts of wild-type (WT) and immune gene mutant larvae.** (A-D) Internal bacterial loads of 2nd (A and C) and 3rd (B and D) instar larva guts from different genotypes of the *Drosophila* host were measured. Bacterial load was expressed in colony-forming units (CFUs). There were no significant differences among the tested groups. The results represent the mean of three biological replicates. No significant differences were noted among the groups marked with the letter “a”. One-way ANOVA, Dunnett’s multiple comparisons test;  $P > 0.05$ . Error bars indicate SEM. Ap, *Acetobacter pomorum*; Lp, *Lactobacillus plantarum*.



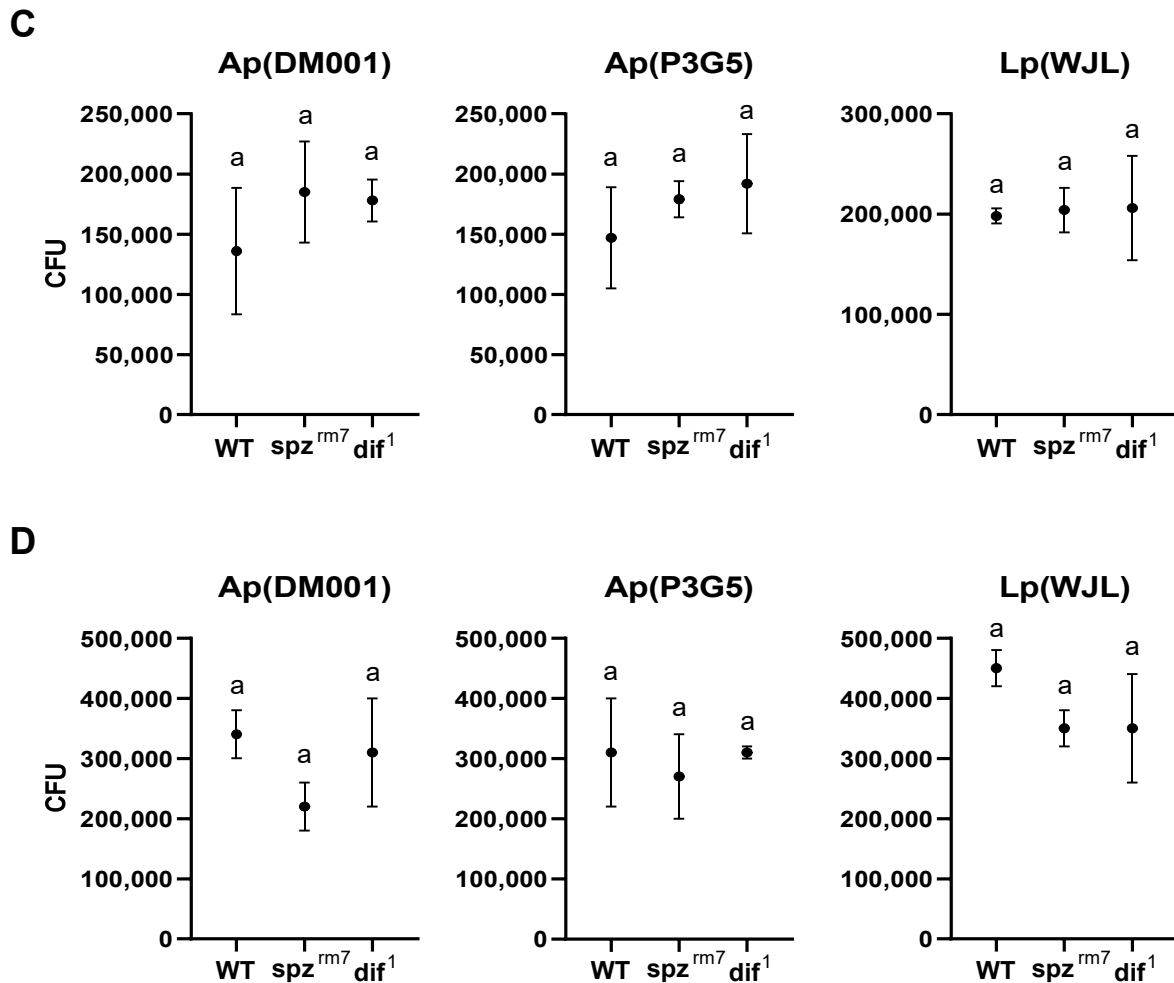


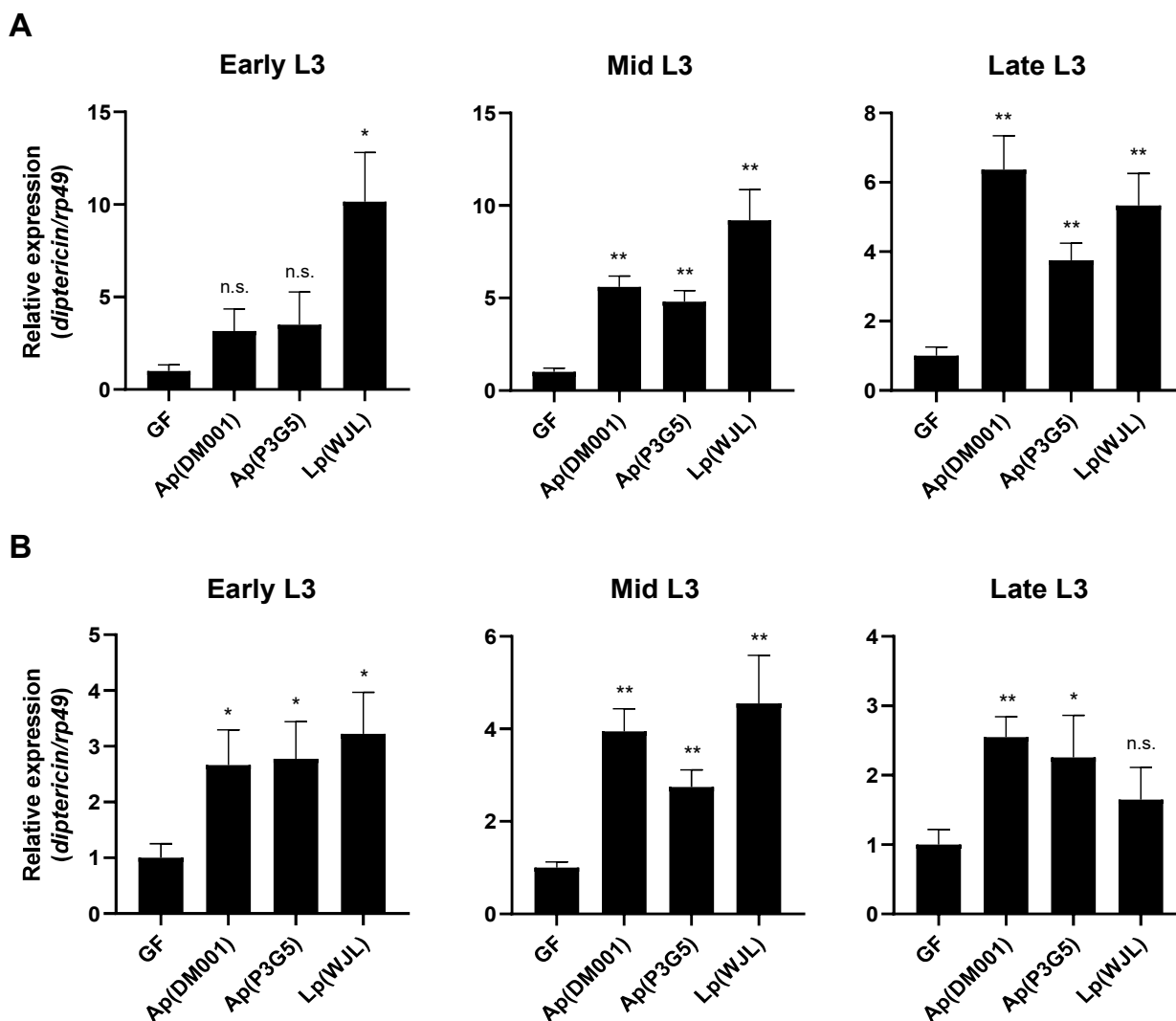
Fig. 4. Continued.

not significantly increased by mutations in Toll pathway genes throughout the larval period (Figs. 4C and 4D), consistent with the notion that in the gut, the Toll immune pathway does not generally play a role in bacterial surveillance. Taken together, these observations indicate that although Ap and Lp could stimulate gut immune activity (see below), activation of the host immune system does not significantly eliminate Ap and Lp in the gut. However, the possibility cannot be excluded that the activation of the IMD pathway modifies the physiology of Ap, blocking the stimulation of host larval development (see Discussion section).

#### IMD immune activity responds to *A. pomorum* and *L. plantarum* similarly in various larval tissues

We investigated whether the specific suppression of the effect of Ap, but not that of Lp, by the IMD pathway might be due to a species-specific stimulation of IMD pathway by Ap and not by Lp. It is known that the activation of the IMD immune pathway leads to the expression of several antimicrobial peptide genes, including *diptericin* (Buchon et al., 2014). Therefore, using *diptericin* expression as a readout for IMD pathway activation, we examined the activity of the IMD

pathway in various larval tissues, including gut, fat body, and body wall muscle. We found that mono-association with Ap or Lp significantly increased the expression of *diptericin* in the gut, without any evidence indicating that Ap stimulated the IMD pathway more than Lp did (Fig. 5A). It should be noted that the stimulation of the IMD pathway by gram-positive bacteria, such as Lp, could be explained by the presence of DAP-type peptidoglycan in Lp (Leulier et al., 2003). Besides the gut, mono-association of Ap or Lp stimulated the expression of *diptericin* also in the fat body, the main organ responsible for systemic immune surveillance (Fig. 5B). In stark contrast, mono-association of neither Ap nor Lp significantly stimulated *diptericin* expression in the body wall muscle (Fig. 5C). Comparison of the extent of IMD immune activation across tissues showed that the IMD immune response was strongest in the gut, suggesting that the gut is the target organ of IMD pathway stimulation by the association of commensal bacteria. Taken together, these observations indicate that the IMD immune pathway is activated to a similar extent by Ap and Lp, rendering it unlikely that the Ap-specific effects on IMD pathway-mediated larval development are due to an Ap-specific stimulation of the IMD pathway.



**Fig. 5. Stimulation of the IMD immune pathway by Lp and Ap in various larval tissues.** (A-C) Mono-association of Ap or Lp in GF larvae stimulated the expression of the *dipteracin* gene, the target gene of IMD immune pathway in the midgut (A) and in the fat body (B), but not in the muscle (C). Samples were prepared at 3 time points (early, mid, late) during the 3rd instar larval period. The results represent the mean of five biological replicates. (A) The *P* values for the comparisons with the control group were as follows. In the early L3 panel: Ap(DM001), 0.1306; Ap(P3G5), 0.2138; Lp(WJL), 0.0139. One-way ANOVA ( $F_{[3,16]} = 5.367$ ;  $P = 0.0141$ ), Dunnett's multiple comparisons test:  $*P < 0.05$ . In the mid L3 panel: Ap(DM001), 0.0020; Ap(P3G5), 0.0011; Lp(WJL), 0.0027. One-way ANOVA ( $F_{[3,16]} = 12.94$ ;  $P = 0.0005$ ), Dunnett's multiple comparisons test:  $**P < 0.01$ . In the late L3 panel: Ap(DM001), 0.0018; Ap(P3G5), 0.0024; Lp(WJL), 0.0039. One-way ANOVA ( $F_{[3,16]} = 10.3$ ;  $P = 0.0012$ ), Dunnett's multiple comparisons test:  $**P < 0.01$ . (B) The *P* values for the comparisons with the control group were as follows. In the early L3 panel: Ap(DM001), 0.0498; Ap(P3G5), 0.0466; Lp(WJL), 0.0303. One-way ANOVA ( $F_{[3,16]} = 3.057$ ;  $P = 0.0210$ ), Dunnett's multiple comparisons test:  $*P < 0.05$ . In the mid L3 panel: Ap(DM001), 0.0011; Ap(P3G5), 0.0041; Lp(WJL), 0.0014. One-way ANOVA ( $F_{[3,16]} = 6.615$ ;  $P = 0.0069$ ), Dunnett's multiple comparisons test:  $**P < 0.01$ . In the late L3 panel: Ap(DM001), 0.0053; Ap(P3G5), 0.0494; Lp(WJL), 0.2545. One-way ANOVA ( $F_{[3,16]} = 3.797$ ;  $P = 0.0264$ ), Dunnett's multiple comparisons test:  $*P < 0.05$ ;  $**P < 0.01$ . (C) The actual *P* values for the comparisons with the control group were as follows. In the early L3 panel: Ap(DM001), 0.6810; Ap(P3G5), 0.5140; Lp(WJL), 0.2678. One-way ANOVA ( $F_{[3,16]} = 0.6089$ ;  $P = 0.6219$ ), Dunnett's multiple comparisons test;  $P > 0.05$ . In the mid L3 panel: Ap(DM001), 0.7168; Ap(P3G5), 0.6283; Lp(WJL), 0.5556. One-way ANOVA ( $F_{[3,16]} = 0.1372$ ;  $P = 0.9359$ ), Dunnett's multiple comparisons test;  $P > 0.05$ . In the late L3 panel: Ap(DM001), 0.3409; Ap(P3G5), 0.1384; Lp(WJL), 0.9573. One-way ANOVA ( $F_{[3,16]} = 0.0946$ ;  $P = 0.9616$ ), Dunnett's multiple comparisons test;  $P > 0.05$ . The results represent the mean of five biological replicates. n.s., not statistically significant. Error bars indicate SEM. IMD, immune deficiency; Lp, *Lactobacillus plantarum*; Ap, *Acetobacter pomorum*; GF, germ-free.

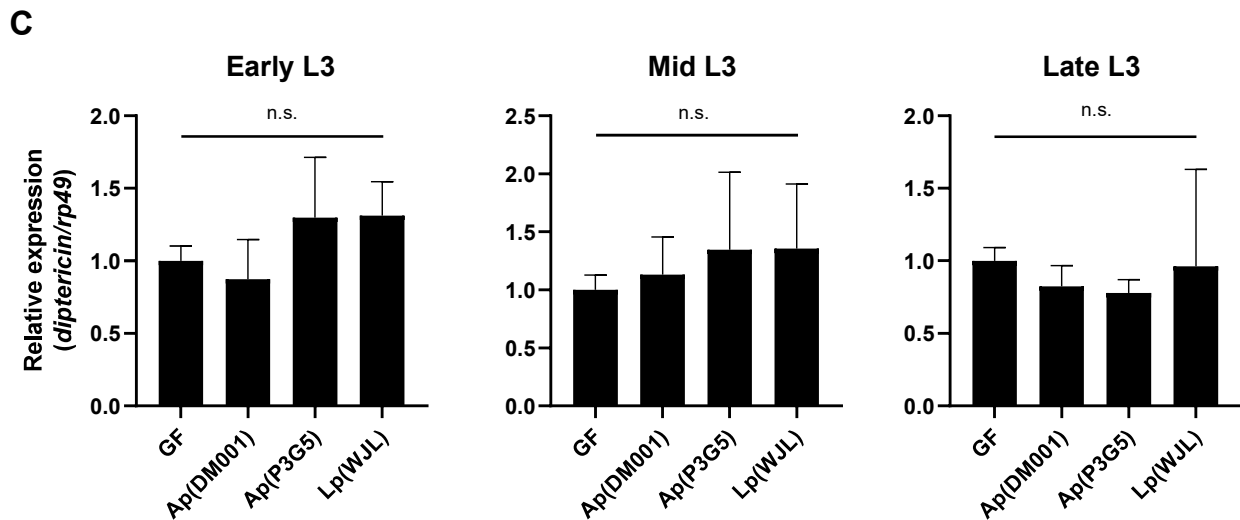


Fig. 5. Continued.

### Expression of peptidase genes in the gut of flies deficient in IMD pathway is similarly induced by *A. pomorum* and *L. plantarum*

As mentioned above, Lp(WJL) has been known to stimulate larval development and growth by increasing the expression of several peptidase genes, thereby enhancing protein digestion in the larval gut (Erkosar et al., 2015; Lee et al., 2020; Matos et al., 2017). Thus, we investigated whether acceleration of Ap-stimulated larval development by IMD pathway mutations might be due to the increased expression of peptidase genes in the gut. Expression levels of three peptidase genes (*jon66Cii*, *jon99Ci*, *CG18179*) known to be increased by Lp(WJL) were dramatically increased in conventionally reared (CR) and Lp(WJL) mono-association groups, as expected (Fig. 6). Interestingly, mono-association of Ap also dramatically increased the expression of the peptidase genes, inducing an effect comparable to those of CR and Lp(WJL) (see Discussion section). Notably, the mutation of IMD pathway genes decreased the peptidase gene expression induced by Lp and Ap (Fig. 6). This result opposes the scenario that IMD pathway mutations accelerate Ap-induced larval development by upregulating peptidase gene expression, but is consistent with the findings of a previous report suggesting that IMD pathway partly mediates Lp-induced peptidase gene expression (Erkosar et al., 2015). These observations indicate that the activity of the IMD pathway is required for sufficient levels of protein digestion induced by commensal bacteria in the gut, which does not contribute to the IMD pathway-mediated suppression of larval development stimulated by Ap.

### Acetic acid and thiamine, the two metabolites of *A. pomorum*, do not mediate the acceleration of larval development induced by mutations of the IMD pathway

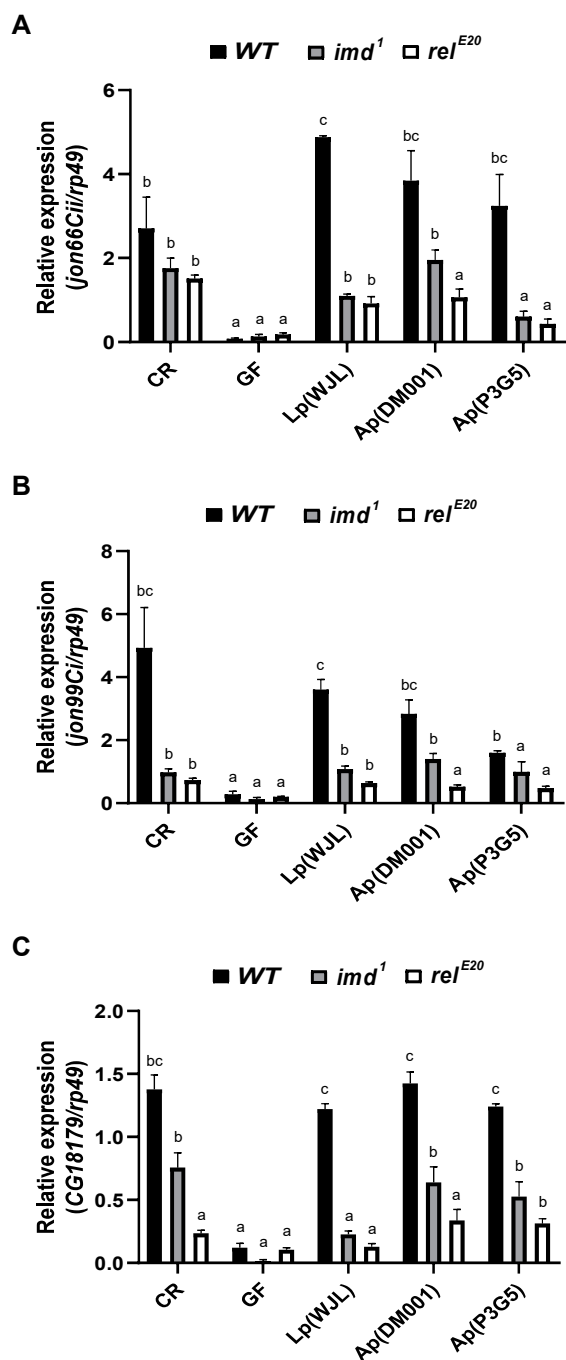
As mentioned earlier, promotion of larval development and growth by mono-association of Ap has been known to be partly mediated by acetic acid, the metabolite generated by PQQ-ADH activity in Ap (Shin et al., 2011). Using the mutant Ap(P3G5) lacking PQQ-ADH activity, we investigated

whether acetic acid participates in the IMD pathway-dependent effects of Ap on larval development. Overall, Ap(P3G5) induced an effect similar to that of wild-type Ap in terms of the IMD-dependent acceleration of larval development (Figs. 1D, 2D, and 3D, Supplementary Fig. S2D). Notably, although Ap(P3G5) failed to stimulate the development of Toll pathway mutant larvae as well as wild-type larvae (Figs. 3A-3C), it did stimulate that of IMD pathway mutants to a similar extent as wild-type Ap (Figs. 1A-1C, Supplementary Fig. S2A-S2C). This result indicates that acetic acid does not play a major role in mediating this phenomenon.

Finally, we explored the possibility that thiamine provided by Ap in the fly diet might be responsible for the IMD pathway-dependent acceleration of larval development by Ap. To this end, we examined whether the addition of thiamine in the fly diet could imitate the effect of Ap mono-association on GF larva. We found that the addition of various amounts of thiamine to the fly diet dramatically stimulated larval development of wild-type GF host, mimicking the effects of Ap mono-association (Fig. 7A), as expected based on the results of a previous report (Sannino et al., 2018). However, the same treatment was found to stimulate the larval development of the IMD pathway-mutant host to a lesser extent than that it stimulated that of the wild-type host (Figs. 7B and 7C). Therefore, the thiamine effect was in the opposite direction to the Ap effect, which accelerates larval development more strongly in IMD pathway-mutant hosts than in wild-type hosts (Fig. 7D). Taken together, these data suggest that the full stimulation of larval development by thiamine requires an intact IMD pathway, and that thiamine, *per se*, does not play a major role in the Ap-mediated, IMD pathway-dependent acceleration of larval development.

### Larval serum protein genes are candidate genes that mediate Ap-dependent developmental stimulation suppressed by IMD pathway

To further probe the molecular mechanism by which IMD pathway deficiency enhances development-promoting



**Fig. 6. Stimulation of peptidase gene expression in the gut by Lp and Ap.** (A-C) Mono-association of Ap or Lp in GF larva stimulated the expression of several peptidase genes (*jon66Cii* in panel A, *jon99Ci* in panel B, and *CG18179* in panel C) in the gut. The robust increase of peptidase gene expression by Lp and Ap was reduced by the mutation of the IMD immune pathway genes. The results represent the mean of five biological replicates. Different letters above the bars indicate significant differences among the groups (one-way ANOVA, Dunnett's multiple comparison test;  $P < 0.05$ ). Error bars indicate SEM. Lp, *Lactobacillus plantarum*; Ap, *Acetobacter pomorum*; WT, wild-type; CR, conventionally reared; GF, germ-free; IMD, immune deficiency.

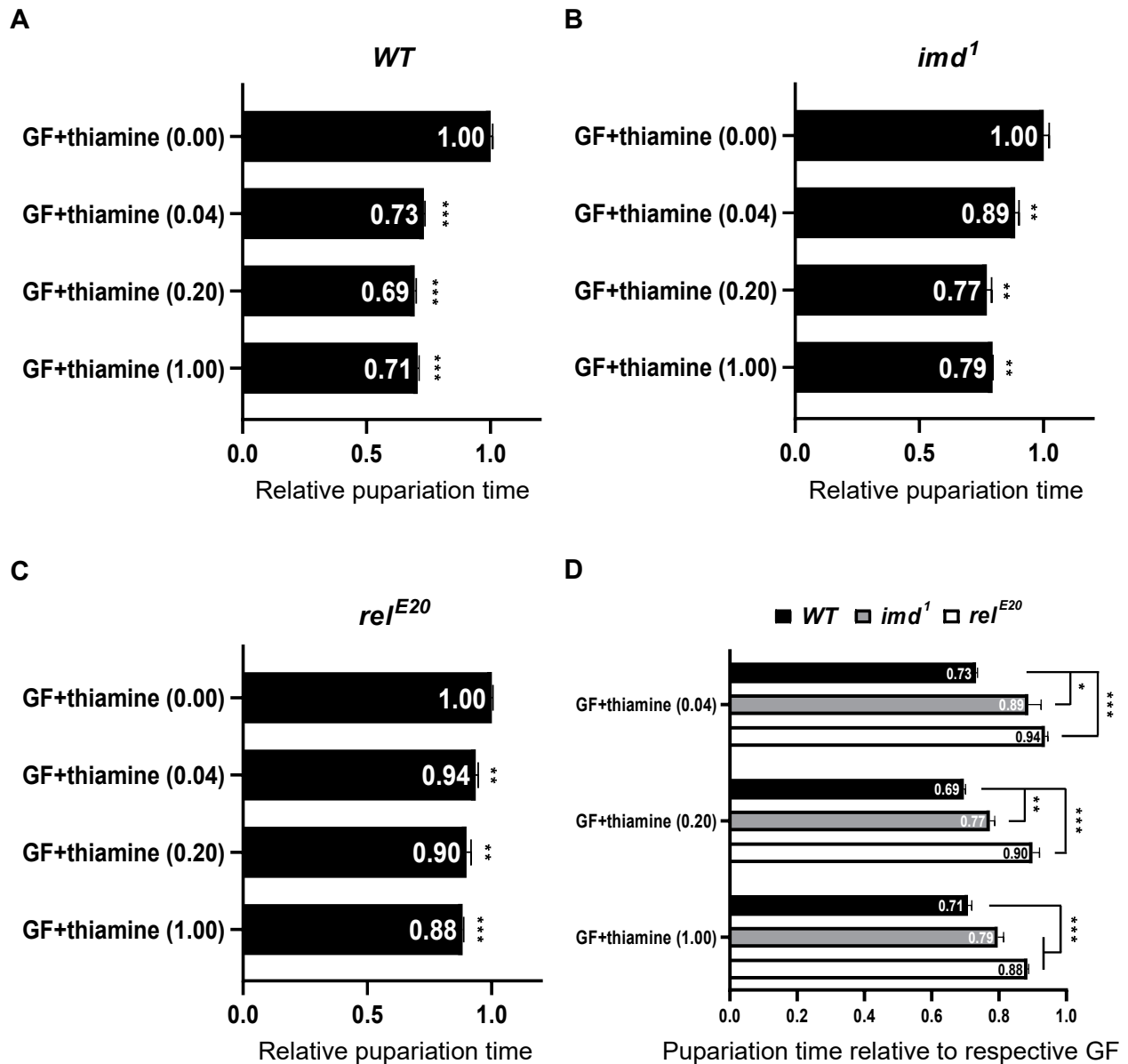
effects of Ap, we performed RNA-sequencing (RNA-seq) analysis of GF and Ap mono-associated larval gut from wild-type and *rel*<sup>E20</sup> flies. We identified 1,120 transcripts that were significantly upregulated by Ap mono-association in wild-type flies. Next, we focused on transcripts that were expressed in higher levels in *rel*<sup>E20</sup> than in wild-type larval gut, obtaining a list of 78 candidate genes (Fig. 8A). Further analyses of the candidate genes based on GO revealed two significantly enriched GO terms, *Lsp* complex of cellular component and nutrient reservoir activity of molecular function (Fig. 8B). The genes involved in these GO terms are closely related with *Lsp* family, consisting of *Lsp1 $\alpha$* , *Lsp1 $\beta$* , *Lsp1 $\gamma$* , and *Lsp2*. *Lsp*s are known as insect hexamerins supplying amino acids and energy for pupation (Benes et al., 1990; Massey et al., 1997; Telfer and Kunkel, 1991) and contribute to various biological processes during insect development (Blackburn et al., 2004; Eliautout et al., 2016; Short et al., 2020).

Given that knockdown of a hexamerin significantly decreases insect developmental rate (Lee et al., 2017), we considered the possibility that enhanced *Lsp* expression induced by Ap mono-association contributes to the advancement of larval development in IMD pathway-mutant fly. Using RT-qPCR, we confirmed the expression levels of *Lsp* genes indicated in the RNA-seq data. The expression levels of four *Lsp* genes (*Lsp1 $\alpha$* , *Lsp1 $\beta$* , *Lsp1 $\gamma$* , and *Lsp2*) were moderately increased in wild-type larval gut following Ap mono-association and strongly increased in *rel*<sup>E20</sup> larval gut following Ap mono-association (Fig. 8C). These findings indicate that the expression of *Lsp* was upregulated, which was repressed by IMD immune pathway.

Finally, we examined whether the increased expression of *Lsp* genes upon Ap mono-association could stimulate larval development. Knockdown of either *Lsp1 $\beta$*  or *Lsp2* specifically in the enterocytes of the larval midgut significantly slowed the larval development of GF larva, indicating *Lsp*s as the positive regulators of the developmental rate (Fig. 8D). Notably, the same genetic manipulation slowed larval development to a greater extent in Ap mono-associated larva than in GF larva (Fig. 8D). Taken together, these results suggest that mono-association of Ap accelerates larval development by upregulating *Lsp* genes, which are repressed by IMD immune pathway (Fig. 8E).

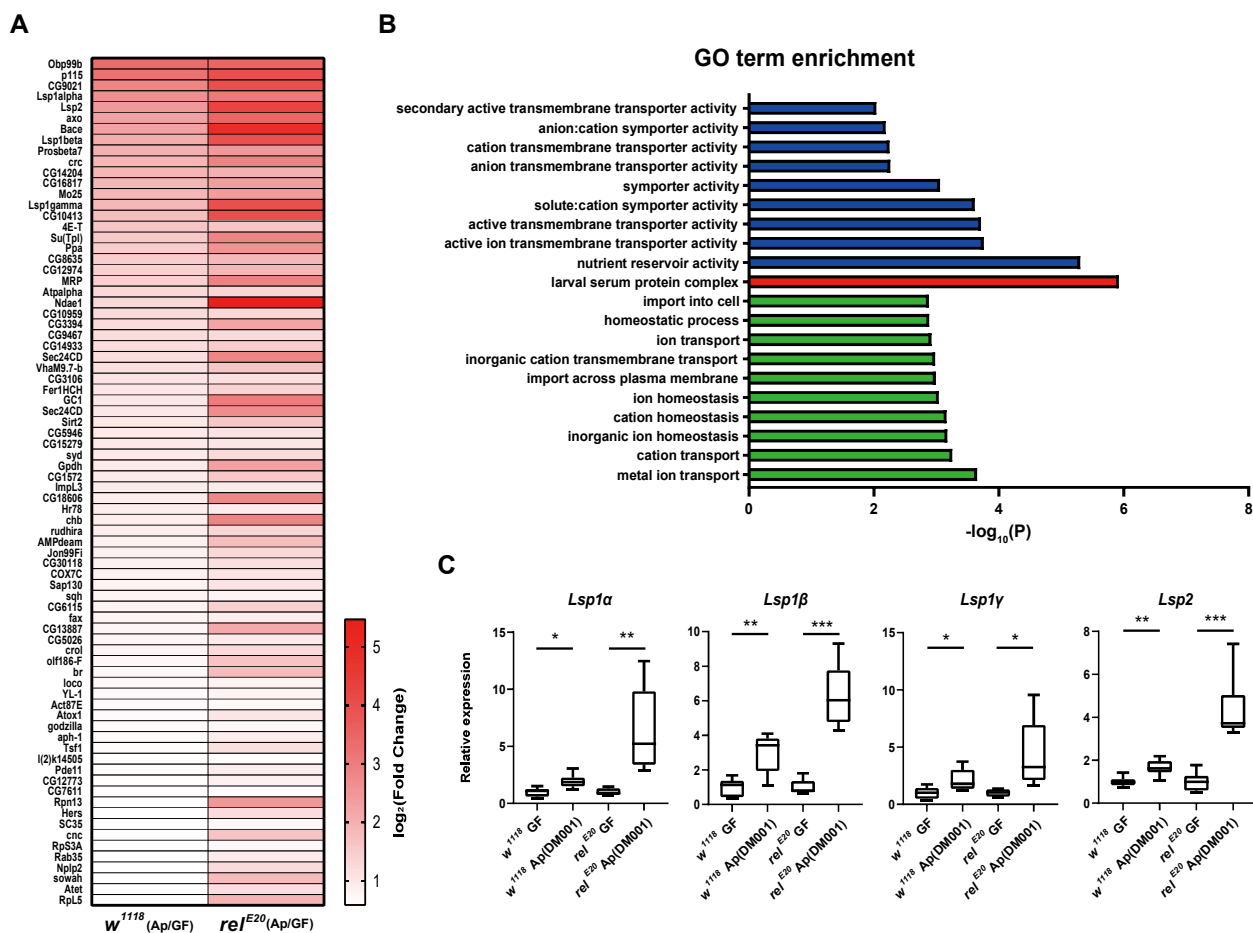
## DISCUSSION

Previous studies on the association of *Drosophila* with commensal bacteria have highlighted the beneficial effects of these bacteria on fly physiology and development. It has been shown that gut microbiota can protect the fly against pathogenic infection. CR and GF flies associated with a strain of Lp are less vulnerable than GF flies not associated with Lp to gut infection by *Serratia marcescens*. This effect is Lp-specific since the association of *Enterococcus faecalis*, another commensal bacterium in *Drosophila*, fails to protect flies against *S. marcescens* infection (Blum et al., 2013). It has been known that gut microbiota influence gut epithelial homeostasis by stimulating a basal level of intestinal stem cell (ISC) activation and subsequent epithelial renewal (Buchon et al., 2009). In the case of host larval development and growth, Ap and



**Fig. 7. Mutation of IMD immune pathway genes fails to accelerate thiamine-induced pupariation.** (A) Addition of thiamine to fly diet strongly advanced the pupariation of GF wild-type (WT) larva. The *P* value for the comparisons with the control group were as follows: thiamine (0.04), 1.92E-05; thiamine (0.20), 1.1E-05; thiamine (1.00), 1.45E-05. One-way ANOVA ( $F_{[3,20]} = 417.2$ ;  $P < 0.0001$ ), Dunnett's multiple comparisons test;  $***P < 0.001$ . (B and C) Addition of thiamine to fly diet advanced the pupariation of GF larva of the *imd* (B) and *rel* (C) mutants. In panel B, the *P* values for the comparisons with the control group were as follows: thiamine (0.04), 0.0019; thiamine (0.20), 0.0020; thiamine (1.00), 0.0012. One-way ANOVA ( $F_{[3,20]} = 32.28$ ;  $P < 0.0001$ ), Dunnett's multiple comparisons test;  $**P < 0.01$ . In panel C, the *P* values for the comparisons with the control group were as follows: thiamine (0.04), 0.0050; thiamine (0.20), 0.0078; thiamine (1.00), 6.35E-05. One-way ANOVA ( $F_{[3,20]} = 20.18$ ;  $P = 0.0004$ ), Dunnett's multiple comparisons test;  $**P < 0.01$ ;  $***P < 0.001$ . (D) Addition of thiamine to the fly diet induced a weaker advancement of the pupariation time of the IMD pathway-mutant larva compared with that of the wild-type larva. The *P* values for the comparisons with the control (WT) group were as follows: thiamine (0.04) in *imd*, 0.0150; thiamine (0.04) in *rel*, 3.94E-05; thiamine (0.20) in *imd*, 0.0080; thiamine (0.20) in *rel*, 0.0007; thiamine (1.00) in *imd*, 0.0005; thiamine (1.00) in *rel*, 1.79E-05. One-way ANOVA, Dunnett's multiple comparisons test;  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ . Pupariation times were normalized to that of GF larva. The results represent six independent observations, each with 30 larvae. Error bars indicate SEM. IMD, immune deficiency; GF, germ-free.





**Fig. 8. Acceleration of larval development by Ap-induced expression of Lsp, which is repressed by IMD immune pathway.** (A) Heatmap representing the transcriptome profile obtained from wild-type and *rel<sup>E20</sup>* larval midgut following Ap(DM001) mono-association. A list of transcripts upregulated to a greater extent by Ap(DM001) mono-association in the larval gut of *rel<sup>E20</sup>* flies compared with wild-type flies included 88 candidate genes (fold change > 1.5). A red bar on the right side of the heatmap denotes the gradient of  $\log_2$  fold change of transcript levels in each fly. (B) Gene ontology (GO) enrichment analysis of upregulated candidate genes shown in panel A, based on three terms including molecular function (blue), cellular component (red) and biological process (green). The bars in the graph mean an enrichment score that was calculated by  $-\log(P)$  value using DAVID software, indicating each term enriched by upregulated genes is statistically significant. (C) *Lsp* expression in the gut was induced by Ap mono-association, which was enhanced in *rel* mutant. The expression of four *Lsp* genes (*Lsp1α*, *Lsp1β*, *Lsp1γ*, and *Lsp2*) slightly increased in wild-type larval gut, but greatly increased in *rel<sup>E20</sup>* larval gut upon Ap(DM001) mono-association. The *P* values for the comparisons with the control group were as follows. In *Lsp1α* panel: Ap(DM001) in wild-type (WT), 0.0103; in *rel<sup>E20</sup>*, 0.0057. In *Lsp1β* panel: Ap(DM001) in WT, 0.0026; in *rel<sup>E20</sup>*, 3.6E-05. In *Lsp1γ* panel: Ap(DM001) in WT, 0.0315; in *rel<sup>E20</sup>*, 0.0211. In *Lsp2* panel: Ap(DM001) in WT, 0.0047; in *rel<sup>E20</sup>*, 0.0004. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001, compared to GF values (*t*-test). The results represent the mean of six biological replicates. (D) Knockdown of *Lsp* in gut enterocytes slowed larval pupariation timing, which was greater when Ap was mono-associated. The *P* values for the comparisons with the control (Myo1A/+) group were as follows. GF in Myo1A>*Lsp1β*-RNAi, 0.0002; in Myo1A>*Lsp2*-RNAi, 6.59E-05. One-way ANOVA ( $F_{[2,15]} = 24.74$ ; *P* < 0.0001), Dunnett's multiple comparisons test; \*\*\**P* < 0.001. Ap(DM001) in Myo1A>*Lsp1β*-RNAi, 1.93E-08; in Myo1A>*Lsp2*-RNAi, 3.69E-08. One-way ANOVA ( $F_{[2,15]} = 165.0$ ; *P* < 0.0001), Dunnett's multiple comparisons test; \*\*\**P* < 0.001. The results represent data obtained from six independent observations, each with 30 larvae. (E) A proposed model showing the relationships between Ap and IMD pathway in regulating the rate of fly larval development. n.s., not statistically significant. Error bars indicate SEM. Ap, *Acetobacter pomorum*; Lsp, larval serum protein; IMD, immune deficiency; GF, germ-free; AMPs, antimicrobial peptides.

Lp, which are the two most common commensal bacterial species in *Drosophila*, have been shown to enhance fly larval development and growth by stimulating hormonal signals mediating growth and maturation. Ap has been shown to

stimulate fly development on a low-nutrition diet by producing metabolites, such as acetic acid and thiamine (Sannino et al., 2018; Shin et al., 2011). Lp has been known to enhance fly development and growth in part by increasing protein

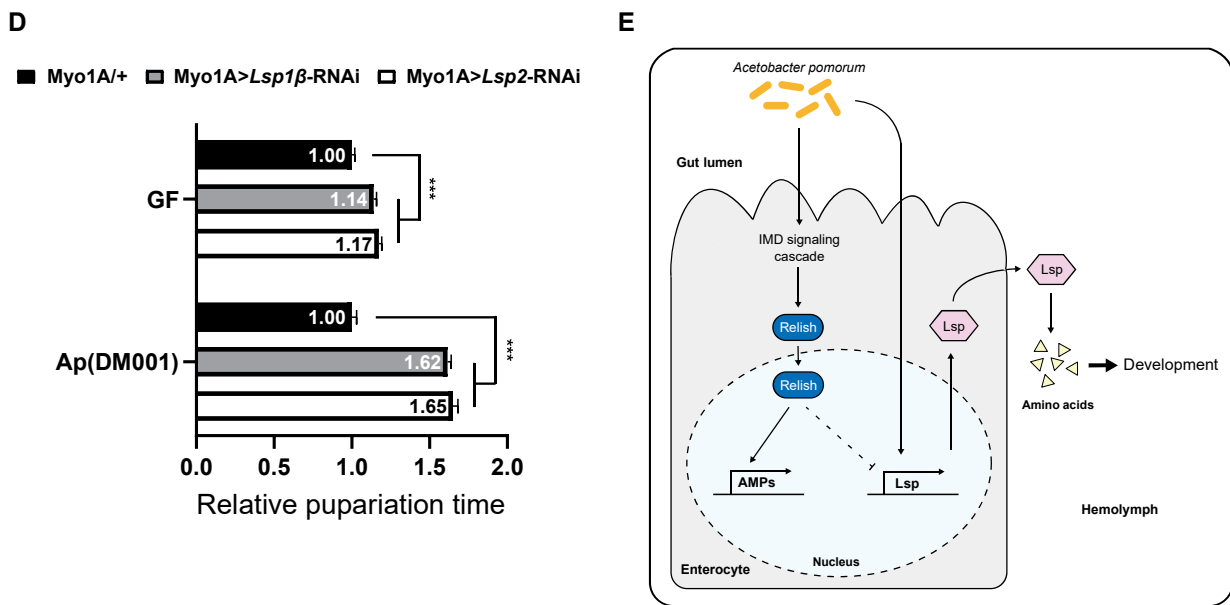


Fig. 8. Continued.

digestion in the gut (Erkosar et al., 2015; Lee et al., 2020; Matos et al., 2017). In this study, we found that mutations in IMD immune pathway genes in *Drosophila* unexpectedly accelerated Ap-stimulated larval development. The effect was Ap-specific because the mutation of IMD immune pathway genes did not accelerate Lp-stimulated larval development. Moreover, mutation of Toll immune pathway did not accelerate the larval development stimulated by the association of commensal bacteria. Although Ap mono-association was found to stimulate the expression of peptidase genes in the gut, this effect was not enhanced by IMD pathway mutations. Acetic acid and thiamine, the two metabolites of Ap known to stimulate fly development (Sannino et al., 2018; Shin et al., 2011), did not mediate the observed interaction between Ap and the IMD immune pathway. Thus, it appears that the IMD immune pathway suppresses Ap-stimulated larval development via an unidentified mechanism. It seems plausible that although it does not eliminate Ap, IMD immune activity may interfere with the microbial production of unknown metabolites besides acetic acid and thiamine that can stimulate host larval development. Alternatively, IMD immune activity might interrupt the host signal transduction pathway that transduces the signal from Ap for the promotion of larval development. RNA-seq and RNAi experiments suggested that *Lsp* genes may be among the candidate factors that mediate Ap-mediated, IMD pathway-dependent developmental stimulation. The identification and characterization of these unknown bacterial metabolites or signaling pathways potentially involving *Lsp*s warrants further investigation.

Another interesting finding of our study was the increase in gut peptidase expression due to the association of Ap or Lp, which confirms a previous observation (Erkosar et al., 2017). Previous studies have showed that Lp(WJL) stimulates host larval development and growth by increasing the expres-

sion of several peptidase genes in the gut, resulting in the efficient digestion and absorption of protein from the diet (Erkosar et al., 2015). Further studies showed that D-alanylation of teichoic acids in the cell wall of Lp as well as the IMD immune pathway of the fly host contribute to the Lp association-induced peptidase expression in the host gut (Matos et al., 2017). Based on the observation that mono-association of Ap can stimulate the activity of IMD immunity in various larval tissues, probably due to the presence of DAP-type peptidoglycan in the cell wall of Ap (Leulier et al., 2003) (Fig. 5), the increase of peptidase expression may be caused by the stimulation of IMD immunity by Ap. This notion is supported by our observation that mutations of the IMD immune pathway reduced the peptidase expression induced by Ap as well as Lp. However, this result refutes the hypothesis that the deficiency of the IMD immune pathway accelerates larval development by enhancing Ap-induced peptidase expression.

In conclusion, our findings on the involvement of previously unrecognized interactions between the IMD immune pathway and Ap commensal bacteria in the regulation of the rate of fly larval development may provide valuable insights for the development of novel techniques of exploiting the beneficial effects of commensal bacteria on host animal health.

Note: Supplementary information is available on the Molecules and Cells website ([www.molcells.org](http://www.molcells.org))

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## AUTHOR CONTRIBUTIONS

J.L., X.S., and B.H. performed experiments. J.L., X.S., and S.H. performed data analysis and prepared the manuscript. C.O.J. provided advice on bacteria handling. S.H. and C.O.J. provided the funding for the experiments.

## CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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