D-alanylation of Teichoic Acids in Bacilli impedes the immune sensing of peptidoglycan in Drosophila

Zaynoun Attieh, Mireille Kallassy Awad, Agnès Rejasse, Pascal Courtin, Ivo Gomperts Boneca, Marie-Pierre Chapot-Chartier, Vincent Sanchis Borja, Laure El Chamy

D-alanylation of Teichoic Acids in *Bacilli* impedes the immune sensing of peptidoglycan in *Drosophila*

Zaynoun Attieh[1,2], Mireille Kallassy Awad[1], Agnès Rejasse[2], Pascal Courtin[2], Ivo Gomperts Boneca[3,4], Marie-Pierre Chapot-Chartier[2], Vincent Sanchis Borja[2] and Laure El Chamy[1]

1- Unité de Recherche Environnement, Génomique et Protéomique, Laboratoire de Biodiversité et Génomique Fonctionnelle, Faculté des sciences, Université Saint-Joseph de Beyrouth- Liban

2- Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France

3- Institut Pasteur, Unité biologie et génétique de la paroi bactérienne, 75724 Paris cedex 15, France

4- INSERM, Equipe Avenir, 75015 Paris, France

Corresponding: laure.chamy@usj.edu.lb;
vincent.sanchis-borja@inra.fr

Abstract

Modification of cell wall components is a prominent mean for pathogens to hinder host immune defenses. Here, using the *Drosophila* model, we aimed at characterizing the role of D-alanine esterification of teichoic acids (TAs) in the resistance of *Bacillus thuringiensis* to host defenses *in vivo*. We show that, by preventing cationic antimicrobial effectors-mediated bacterial lysis, this cell wall modification also limits the release of peptidoglycan immunostimulatory fragments thus impeding their sensing and the subsequent induction of the IMD- NF-κB pathway. Interestingly, we show that this strategy is also adopted by *Lactobacillus plantarum*, a *bona fide* commensal, to fine-tune its immunomodulatory potential in the *Drosophila* gut. Markedly, we show that the D-alanylation of TAs is essential for the resistance of *L. plantarum* to gut lysozyme. Altogether our data shed light on the mechanism underlying the persistence and the low immunostimulatory potential of *L. plantarum* in the *Drosophila* gut.

Introduction

The external surface of microorganisms is a central platform for interactions with multicellular hosts. Evolutionarily selected Pattern Recognition Receptors (PRRs) of the innate immune system sense key microbial surface molecules such as fungal glucans, Gram-negative bacterial lipopolysaccharide (LPS), Gram-positive bacterial Peptidoglycan (PGN) and Teichoic Acids (TAs) to alert the host of the microbial non-self and to initiate immune responses (Medzhitov, 2007). PRRs, such as the Toll-like receptors and NOD-like receptors act at the forefront of the mucosal immune barrier. They control the expression of several immune induced genes such as antimicrobial peptides (AMPs) and cytokines, the differential profiles of which dictate the outcome of the ensued immune response (Hold, Mukhopadhya, & Monie, 2011). Therefore, several hypotheses consider microbial surface molecules as plausible candidates for effector probiotic molecules (Kleerebezem et al., 2010). Likewise, several pathogens have evolved specialized mechanisms to modify their external surface in order to avoid detection or to develop resistance to potent, highly conserved, immune effectors such as AMPs and lysozymes (Anaya-Lopez, Lopez-Meza, & Ochoa-Zarzosa, 2013; Bechinger & Gorr, 2017; Koprivnjak & Peschel, 2011; Ragland & Criss, 2017). In humans, both lysozyme and AMPs are expressed at surface epithelial cells as well as in phagocytes and thus act at the frontline of the host defenses (Dommett, Zilbauer, George, & Bajaj-Elliott, 2005). Most AMPs are small cationic molecules with a pronounced amphipathy, a property that accounts, as commonly agreed, for their membrane targeting microbicidal activity. According to the model, AMPs are preferentially attracted to negatively charged surfaces of the microbial cell envelopes where they get embedded into the hydrophobic regions of the lipid membranes, thereby leading to membrane destabilization and ultimately to cell death (Joo, Fu, & Otto, 2016). Lysozyme comprises an evolutionarily conserved family of bactericidal proteins that are found in species ranging from bacteriophages to man (Dommett et al., 2005). Their canonical killing mechanism relies on their

muramidase activity, which cleaves the β-(1,4)-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine moieties of the bacterial cell wall peptidoglycan. Resulting loss of cell wall integrity leads to rapid cell lysis and death (Callewaert & Michiels, 2010; Ragland & Criss, 2017). The cationic nature of lysozyme is very important for bactericidal activity since it favors its binding to the negatively charged bacterial cell envelop and hence access to the PGN substrate.

In addition to the thick layer of PGN, the cell wall of Gram-positive bacteria is characterized by the presence of highly charged anionic polymers of repeating alditol phosphate residues called Teichoic acids (TAs). TAs represent the most abundant component of Gram-positive bacterial cell wall and play crucial roles in their pathogenesis (Brown, Santa Maria, & Walker, 2013; Joo et al., 2016). Notably, modification of TAs by addition of positively charged D-alanine esters residues reduces their net negative charge thus lowering the attraction of cationic antimicrobial peptides to the bacterial cell wall. This process is performed by the gene products of the *dlt* operon that is highly conserved in most Gram-positive bacteria including probiotic and pathogenic species (Abachin et al., 2002; Abi Khattar et al., 2009; Heaton & Neuhaus, 1992; Kristian et al., 2005; Perego et al., 1995; Peschel et al., 1999). According to currently available genome sequences, the *dlt* operon comprises four to five genes, *dltXABCD* (Abi Khattar et al., 2009; Kamar et al., 2017; Perego et al., 1995). DltA is a D-alanine-D-alanyl carrier protein ligase that catalyzes the D-alanylation of the D-alanyl carrier protein DltC (Debabov et al., 1996; Heaton & Neuhaus, 1992, 1994). The roles of DltX, DltB and DltD are less clear (Debabov, Kiriukhin, & Neuhaus, 2000; Kamar et al., 2017; Neuhaus & Baddiley, 2003). Nevertheless, in all tested Gram-positive bacteria species, genetic disruption of any of the five genes of the *dlt* operon completely abrogates its function. Compared to wild-type strains, *dlt* mutants have a higher negative charge on the cell surface and are significantly susceptible to cationic antimicrobial effectors including AMPs and lysozyme (Fabretti et al., 2006; Kovacs et al., 2006;

Kristian et al., 2005; Kristian et al., 2003; Perea Velez et al., 2007). In addition, *in vivo* studies clearly show a significantly attenuated virulence of the *dlt* mutants compared to wild-type strains of many human pathogens (Abachin et al., 2002; Abi Khattar et al., 2009; Fisher et al., 2006; Perego et al., 1995; Peschel et al., 1999; Poyart et al., 2003). However, despite deep interest in the characterization of the function of the *dlt* operon, our understanding of its exact role in the bacterial resistance to the innate immune system *in vivo* remains limited.

Drosophila has long emerged as a well-suited model for the study of host-pathogen interactions (Ferrandon, 2013; Lemaitre & Hoffmann, 2007). The hallmark of the *Drosophila* host defense is the immune-induced expression of genes encoding potent AMPs (Ferrandon, Imler, Hetru, & Hoffmann, 2007; Lemaitre & Hoffmann, 2007). These peptides are synthesized by the fat body cells (the fly immune organ) and secreted into the hemolymph where they constitute the main effectors of the humoral systemic response. AMPs are also produced locally at the epithelial barriers such as the gut, where they act synergistically with ROS to limit the infections (Ferrandon, 2013; Imler & Bulet, 2005). Two highly conserved NF-κB signaling cascades, the Toll and the Immune Deficiency (IMD) pathways, control the expression of AMP genes. Whereas both pathways contribute to the regulation of the systemic immune response, only the latter regulates the expression of AMP genes in the gut through the activation of the NF-κB transcription factor Relish (El Chamy, Matt, Ntwasa, & Reichhart, 2015; Ferrandon, 2013). The IMD pathway is elicited upon the sensing of DAP-type PGN fragments by members of the Peptidoglycan Recognition Protein (PGRP) receptors family; the membrane bound PGRP-LC and the intracellular PGRP-LE (Chang, Chelliah, Borek, Mengin-Lecreulx, & Deisenhofer, 2006; Chang et al., 2005; Choe, Lee, & Anderson, 2005; Choe, Werner, Stoven, Hultmark, & Anderson, 2002; Gottar et al., 2002; Kaneko et al., 2004; Kaneko et al., 2006; Leulier et al., 2003; Mellroth & Steiner, 2006; Ramet, Manfruelli, Pearson, Mathey-Prevot, & Ezekowitz, 2002). Potential immune effectors, such as lysozymes are also expressed at the epithelial barrier

(Daffre, Kylsten, Samakovlis, & Hultmark, 1994). However, their global contribution to the host defense and intestinal homeostasis remains obscure. More recently, *Drosophila* has become a powerful model for the characterization of the molecular mechanisms underlying the mutualistic host-microbiota interactions (Erkosar & Leulier, 2014; W. J. Lee & Brey, 2013; Ma, Storelli, Mitchell, & Leulier, 2015)**.** Indeed, several studies have proven the relative simplicity of the fly intestinal microbiota, which comprises approximately 30 phylotypes with a major representation of *Lactobacillaceae* and *Acetobacteraceae* (Blum, Fischer, Miles, & Handelsman, 2013; Chandler, Lang, Bhatnagar, Eisen, & Kopp, 2011; Corby-Harris et al., 2007; Cox & Gilmore, 2007; Staubach, Baines, Kunzel, Bik, & Petrov, 2013; C. N. Wong, Ng, & Douglas, 2011). Reports have begun to illustrate the impact of this microbial community on the *Drosophila* host biology (Buchon, Broderick, Chakrabarti, & Lemaitre, 2009; Iatsenko, Boquete, & Lemaitre, 2018; Jones et al., 2013; Sharon et al., 2010; Shin et al., 2011; Storelli et al., 2011; A. C. Wong, Dobson, & Douglas, 2014). Notably, the gut commensals largely influence the *Drosophila* midgut transcriptome and promote the expression of genes associated with gut physiology and metabolism as well as tissue homeostasis and immune defenses (Broderick, Buchon, & Lemaitre, 2014; Erkosar & Leulier, 2014). Interestingly, some *Lactobacillus plantarum* strains recapitulate the benefits of a *bona fide Drosophila* commensal thus providing a simple model for the investigation of the intricate mechanisms underlying the impact of intestinal bacteria on host physiology and mucosal immunity (Jones et al., 2013; Storelli et al., 2011). In this context, high throughput analyses have shown that the gut-associated bacteria set an immune barrier in the *Drosophila* gut through the activation of a transcriptional program that is largely dependent on the IMD pathway (Bosco-Drayon et al., 2012; Broderick et al., 2014; Buchon et al., 2009; Ryu et al., 2008). Remarkably though, the expression of AMP genes remains at its basal level compared to that induced upon an oral infection (Bosco-Drayon et al., 2012) and any alteration in this transcriptional profile leads to commensal dysbiosis, which dramatically affects the host fitness

and lifespan (Bonnay et al., 2013; Broderick et al., 2014; Ryu et al., 2008). Recent studies have shown that the immune response is compartmentalized in the *Drosophil*a gut (Bosco-Drayon et al., 2012; Buchon, Broderick, & Lemaitre, 2013) and that multiple negative regulators intervene at different levels of the IMD pathway in order to fine-tune its activation (Aggarwal & Silverman, 2008; Bischoff et al., 2006; Guo, Li, & Lin, 2014; Kleino et al., 2008; Lhocine et al., 2008; Mellroth, Karlsson, & Steiner, 2003; Paredes, Welchman, Poidevin, & Lemaitre, 2011; Ryu et al., 2004; Zaidman-Remy et al., 2006). Among these negative regulators are catalytic members of the PGRP family, such as PGRP-SC and PGRP-LB, which degrade PGN fragments into non-immunostimulatory moieties and the intracellular protein, Pirk, which binds to the PGRP-LC and PGRP-LE receptors thus disrupting downstream signaling complex (Aggarwal et al., 2008; Kleino et al., 2008; Kleino et al., 2017; Lhocine et al., 2008). However, similarly to infectious bacteria, *L. plantarum* and the major elements of the fly microbiota produce DAP-type PGN and most of the IMD negative regulators act in a feedback loop. Thus, what makes this microbial community only a mild inducer of the AMP genes and how it is tolerated in the *Drosophila* gut remains intriguing (Bosco-Drayon et al., 2012).

Here, using the *Drosophila* model, we aimed at a better characterization of the intricate *dlt*-dependent mechanisms that allow bacteria to resist the innate immune response *in vivo*. Our results show that, D-alanylation of TAs impedes the sensing of PGN from *B. thuringiensis* in *Drosophila* thus hampering the activation of the IMD pathway upon a systemic infection. This mechanism is also used by *L. plantarum* to fine-tune its induced immune response in the *Drosophila* gut. In particular, we show that the D-alanylation of TAs is essential for the resistance of *L. plantarum* to intestinal lysozyme and thus for its persistence a core component of the *Drosophila* microbiota but also for the modulation of its ensued NF-κB-dependent epithelial immune response.

Results

Beyond resistance to AMPs, D-alanylation of TAs in *Bacilli* prohibits the systemic activation of the *Drosophila* IMD pathway

We have recently shown that *B. thuringiensis* is highly virulent to adult *Drosophila* in a septic injury infection model. Using a *dltX* mutant of *B. thuringiensis 407* Cry$^-$ strain (*Bt407ΔdltX*), we showed that this phenotype is completely dependent on the D-alanylation of its TAs, which confers resistance to the IMD-dependent humoral immune response (Kamar et al., 2017). Here, we assess whether this phenotype is due to the resistance of *Bt407* to cationic AMPs *in vivo*. For that, we resorted to a rescue experiment of the susceptibility of adult flies to the infections by the wt and the *dlt* mutants of *Bt407 via* the overexpression of *Cecropin*, an-IMD responsive gene encoding a cationic AMP. *Cecropin* overexpression was driven by the UAS-Gal4 system in *Drosophila* using the fat-body specific driver c564-Gal4 (Harrison, Binari, Nahreini, Gilman, & Perrimon, 1995) (Figure 1 – figure supplement 1). In addition to our previously published *Bt407ΔdltX* mutant, our survival experiments employed a full *dlt*-operon deletion mutant, *Bt407Δdlt*, which we generated by allelic exchange with a kanamycin resistance cassette (see experimental procedures). Loss of function of the *dlt* operon was confirmed by quantification of D-Alanine esterified to TAs by HPLC analysis, which proved impaired D-alanylation of TAs in the *Bt407Δdlt* mutant as compared to the wt strain (Figure 1 – figure supplement 2). The survival curves of infected adult flies presented in Figure 1 clearly show that *Bt407* is highly virulent to both wt and *relish* mutant flies whereas the *Bt407ΔdltX* and *Bt407Δdlt* mutants are only pathogenic to the latter (Figure 1 A and C). In addition, the similar susceptibility of wt flies and *relish* mutants to the *Bt407* infection is mirrored by the rate of bacterial growth in the hemolymph of the infected flies (Figure 1 B and D). The bacterial loads are equivalent to those retrieved from *relish* mutants upon an infection with *Bt407Δdlt* and *Bt407ΔdltX* (Figure 1 B and D). These results confirm that the D-alanylation of TAs confers *Bt407* resistance to the systemic

176 humoral immune response in *Drosophila*. Moreover, whereas the overexpression of *Cecropin*
177 does not alter the virulent phenotype of *Bt407*, it significantly ameliorates the survival of the wt
178 flies and completely rescues the phenotype of the *relish* mutants when infected with the
179 *Bt407ΔdltX* and *Bt407Δdlt* (Figure 1A and C). These results correlate with the cessation of
180 bacterial growth in the hemolymph of surviving infected flies (Figure 1 B and D). Altogether,
181 these results indicate that D-alanylation of TAs in *Bt407* confers resistance to Cecropin *in vivo*.
182 This phenotype is strictly dependent on the activity of the *dlt* operon as complementation of the
183 *Bt407ΔdltX* mutant with the *dltX*-ORF (*Bt407ΔdltΩdltX)* completely restores a virulence similar
184 to that of the wt *Bt407* strain (Figure 1 – figure supplement 3).

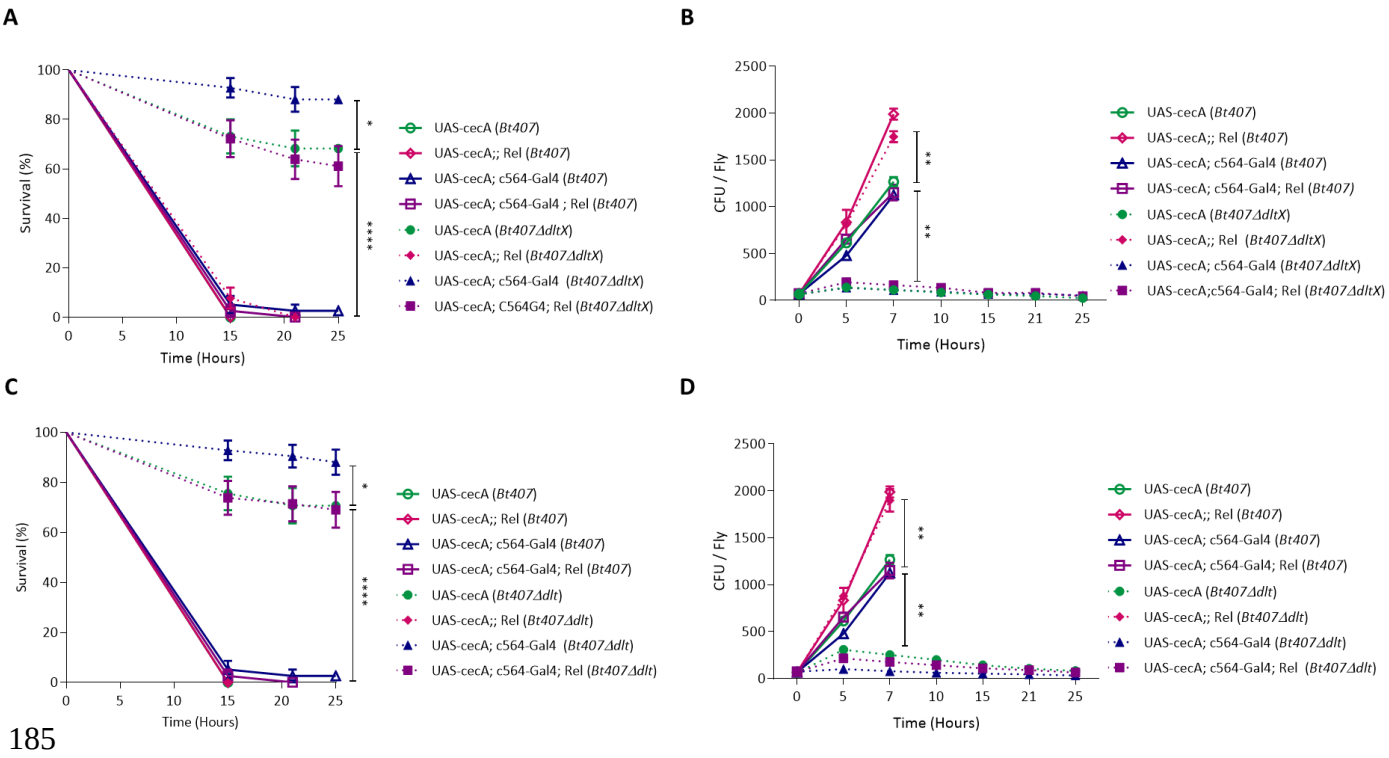

Figure 1: D- alanylation of TAs confers *Bacillus thuringiensis* resistance to the AMP-dependent systemic immune response in *Drosophila*

(A and C) Survival of adult wild-type (wt) or relish (*Rel*) mutant, overexpressing or not a *Cecropin A* transgene (under the control of the c564-Gal4 fat body driver) to an infection with *Bt407*, *Bt407ΔdltX* (A) and *Bt407Δdlt* (C). (B and D) Internal bacterial loads retrieved from adult flies infected with *BT407*and *Bt407ΔdltX* (B) or Bt407*Δdlt* (D). The Colony Forming Unit (CFU) counting was performed only on survival flies.
Data are pooled from three independent experiments (mean and s.d.). Statistical tests were performed using the Log Rank test for the survival assays and Mann-Whitney test for the CFU counting within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).The exact p values are listed in Figure 1 – source data 1.

The following source data and figure supplements are available for figure 1:
Source data 1. *p* values for figure 1
Figure supplement 1. Overexpression of *CecropinA* gene.
Figure supplement 2. Amounts of D-alanine released from whole cells by alkaline hydrolysis for the wild type and the *dlt* mutant strains of *Bt407*.
Figure supplement 3. Complementation of the *Bt407ΔdltX* mutant with the *dltX-ORF* (*Bt407ΔdltΩdltX*) completely restores its virulence similarly to the wt *Bt407* strain.

Since the D- alanylation of TAs conveys a modification of the bacterial surface, we assessed whether it affects the sensing of *Bt* by the *Drosophila* innate immune system. Hence, in order to compare the activation of the IMD pathway by *Bt407* and the Δ*dlt* mutants, we infected the flies by septic injury and quantified the expression of *Diptericin*, an AMP-encoding gene, four hours following infection. The *Bt*- triggered *Diptericin* expression was compared to that induced by *Escherichia coli*, a conventionally used microbial inducer of the IMD pathway (Dushay, Asling, & Hultmark, 1996; Hedengren et al., 1999; Kaneko et al., 2004; Leulier et al., 2003). As shown in Figure 2A, *Bt407* is only a mild-inducer of the IMD pathway as compared to *E. coli*. However, both *Bt407ΔdltX* and *Bt407Δdlt* infection result in *Diptericin* expression level similar to that induced by *E. coli*. This response is strictly dependent on the activation of the IMD pathway since it is abolished in *PGRP-LC* and *relish* mutant flies. The phenotype is completely reverted in the *Bt407ΔdltX* complemented strain (*Bt407ΔdltXΩdltX*) (Figure 2A). Similar results were obtained from another read-out of the IMD pathway, *Attacin D* (Figure 2B). Altogether, our results indicate that beyond resistance to AMPs, the D-alanylation of TAs impedes the sensing of *Bt407* by the innate immune system, thus significantly reducing its immune-stimulatory potential and further contributing to its pathogenicity in *Drosophila*.

Next, we asked whether this mechanism of *dlt*-dependent camouflage of *Bt* is also used by other *Bacilli* species. Therefore, we checked for the immune induction of the IMD response in flies infected with a wt *Bacillus subtilis* (*B. subtilis*) and its *dlt* mutant (*B. subtilisΔdlt*). As shown in figure 2C, the *Diptericin* expression profile elicited by these bacterial strains is equivalent to that activated upon the infection of the flies by *Bt407* and the *Bt407Δdlt* mutant respectively. These data suggest that the *dlt* function results in a restricted elicitation of the IMD pathway also in *Bacilli*. To exclude that the limited induction of the IMD pathway results from its active inhibition from live *Bacilli,* we repeated these experiments using heat killed bacteria and found similar results (Figure 2D and 2E). We conclude that the D-alanylation of TAs highly masks PGN from sensing by PGRP-LC.

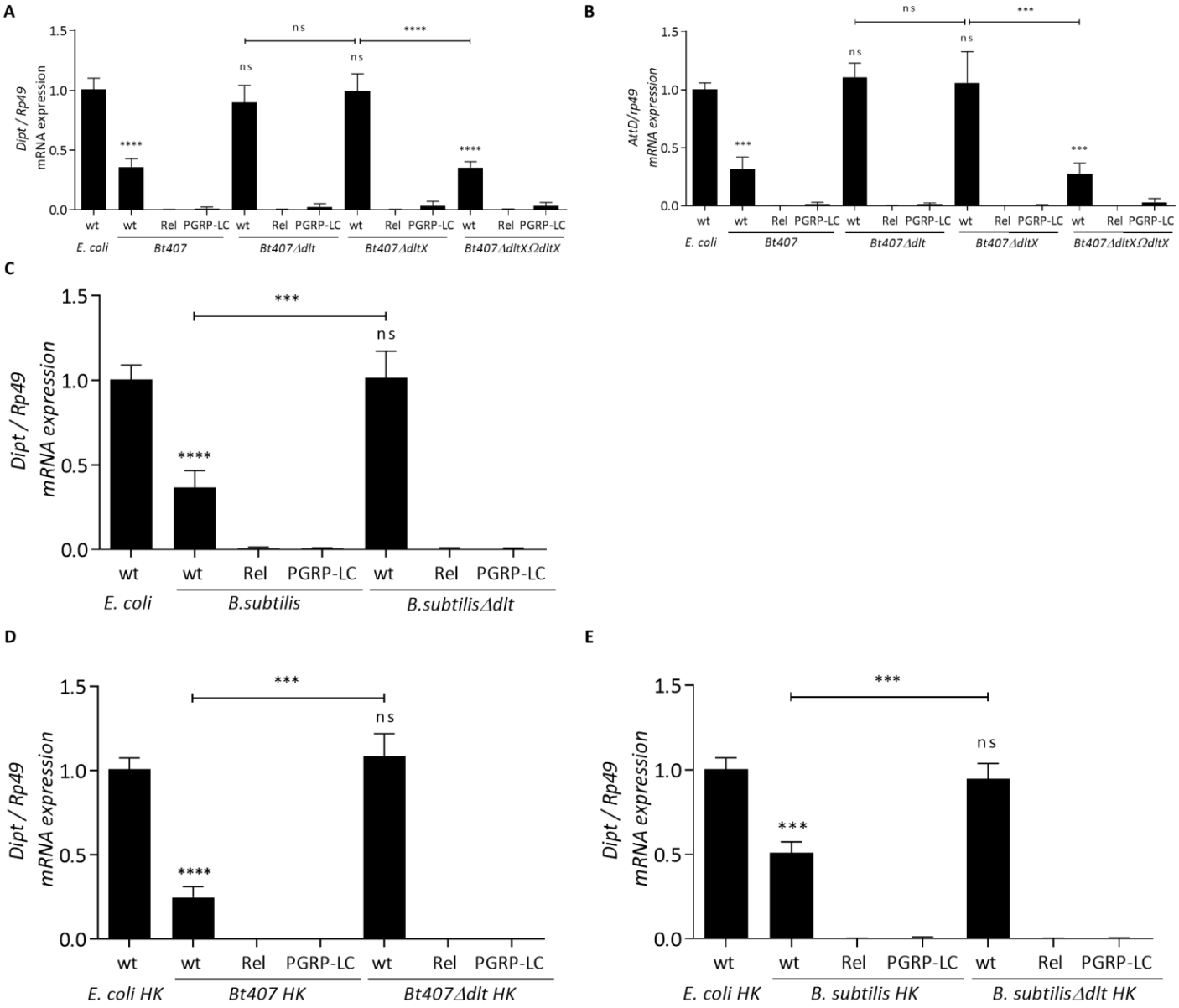

Figure 2: The D- alanylation of TAs confines the induction of the IMD pathway upon a systemic infection of *Drosophila* by *Bacilli*.

Relative expression of the *Diptericin* (*Dipt*) (A, C D and E) or *Attacin D* (*AttD*) (B) transcripts in wild-type (wt) or *relish* (*Rel*) mutant flies induced by living or heat-killed *Bt407, Bt407Δdlt, Bt407ΔdltX* and *Bt407ΔdltXΩdltX (A, B and D) or B. subtilis (C and E).* Transcripts expression was measured by RT-qPCR in total RNA extracts 4 hours upon the induction. Ribosomal protein 49 (Rp49) transcript was used as reference gene. Transcripts levels are compared to that triggered in wt flies infected by *E. coli* as a control.
Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).The exact p values are listed in Figure 2 – source data 1.

The following source data is available for figure 2:
Source data 1. p values for figure 2

D-alanylation of TAs is essential for the persistence of *Lactobacillus plantarum* in the *Drosophila* gut and the modulation of its immune-stimulatory potential as a core component of the gut-associated microbiota

In keeping with the role of D-alanine esterification of TAs in the resistance of *Bacilli* to antimicrobial effectors and the dampening of their IMD- eliciting potential, we asked whether this phenomenon would also account for the intestinal immune-tolerance of *L. plantarum* as a prevalent member of *Drosophila* microbiota. Therefore, we first determined whether the D-alanylation of TAs is required for the colonization and the persistence of *L. plantarum* in the *Drosophila* gut. The previously characterized *L. plantarum*^{WJL} strain (*Lp*^{WJL}), which was isolated from the intestine of *Drosophila melanogaster*, has a low transformation efficiency, which makes it difficult for site directed mutagenesis of the *dlt* operon. Therefore, we used the *L. plantarum*^{WCFS1} strain (*Lp*^{WCFS1}), isolated from human saliva, and its previously characterized *dlt* mutant (*Lp*^{WCFS1}*Δdlt*)(Hayward & Davis, 1956) (Kleerebezem et al., 2003; Palumbo et al., 2006). In a recent study, Newell *et al.* have reported that the *Lp*^{WCFS1} strain shares the same ability as the *Lp*^{WJL} strain to colonize the *Drosophila* gut (Newell et al., 2014). These data were further confirmed in our hands (Figure 3 – figure supplement 1) thus; we checked whether *Lp*^{WCFS1} reproduced the same *Lp*^{WJL} triggered IMD-dependent immune response when introduced in the flies. For that, adult flies were fed on a mixture of antibiotics for three days to generate gut germ free flies (GF) (Stein, Roth, Vogelsang, & Nüsslein-Volhard), which were then recolonized with physiological quantities of the different strains of *L.* plantarum (Bonnay et al., 2013; Storelli et al., 2011). GF and recolonized states were attested by plating the dissected guts on appropriate agar media. The comparison of the *Diptericin* expression levels in the guts of flies conventionally reared on cornmeal agar medium (CR) to that of GF flies, shown in Figure 3 – figure supplement 1, indicates that the gut microbiota sets an IMD- dependent immune response. This immune response is mildly but significantly induced in guts recolonized with *Lp*^{WJL} and

Lp^{WCFS1} as compared to GF guts. These results are in line with data previously reported by Bosco-Drayon *et al.* showing that *Lp*^{WJL} is a mild inducer of AMP genes in the gut. These authors also reported that *Lp*^{WJL} most potently activates the expression of genes encoding negative regulators of the IMD pathway such as *pirk* and *PGRP*-SC1. These results were also confirmed for the *Lp*^{WCFS1} strain (Figure 3 – figure supplement 1). Collectively, our results attest that *Lp*^{WCFS1} reproduces the IMD-dependent immune response activated by the *Drosophila* native gut associated *Lp*^{WJL} strain. Therefore, we pursued our analysis by checking whether the *dlt* operon is required for the persistence of *L. plantarum* in the *Drosophila* gut. According to recent data, the *Drosophila* microbial community is sustained by continuous replenishment through feeding (Blum et al., 2013). Likewise, work from Leulier's laboratory have previously shown that upon association in gnotobiotic flies, *L. plantarum* colonizes the *Drosophila* midgut and its external culture medium and resists the passage through the digestive tract of its host (Storelli et al., 2011). Therefore, in order to evaluate the persistence of the wt and *dlt* mutant strains in the *Drosophila* gut, GF adult flies were monoassociated by feeding on *Lp*^{WCFS1} or the *Lp*$^{WCFS1}\Delta dlt$ mutant strains for three days and then transferred to fresh cornmeal agar medium vials. The bacterial loads in the fly guts were assessed on daily basis. As shown in Figure 3A, the bacterial count remained invariable in the guts of wt flies mono-associated with Lp^{WCFS1} whereas it significantly declined in the guts of the flies recolonized by the Lp$^{WCFS1}\Delta dlt$ mutant. In contrast, the bacterial loads of both wt and *Lp*$^{WCFS1}\Delta dlt$ mutants remained invariable in the guts of mono-associated *relish* mutants (Figure 3A). These results suggest that the D-alanylation of TAs is required for the resistance of *L. plantarum* to the IMD-dependent immune response and its subsequent persistence in the *Drosophila* gut. To further check whether this persistence particularly stems from the *dlt*-dependent resistance of *L. plantarum* to cationic antimicrobial effectors, we examined the persistence of the wt and *dlt* mutant strains of *Lp*^{WCFS1} in the guts of transgenic flies overexpressing *Cecropin* (Figure 3 – figure supplement 2). As shown in Figure

3A, the counts of the wt *Lp*^{WCFS1} strain were constant in the guts of the recolonized flies despite the overexpression of Cecropin, whereas those of the *Lp*$^{WCFS1\Delta dlt}$ mutant strain dramatically declined in less than four days. Altogether, these data underline a role of the *dlt* operon in the resistance of *Lp*^{WCSF1} to cationic antimicrobial effectors thus favoring its persistence in the *Drosophila* gut.

Next, we questioned whether the D-alanylation of TAs influenced the immune-stimulatory potential of *L. plantarum* in the intestine. To this end, we compared the intensity of the IMD response elicited in the guts of GF flies monoassociated with either *Lp*^{WCFS1} or *Lp*^{WCFS1}*Δdlt*. As shown in Figure 3B, the *Lp*^{WCFS1}*Δdlt* mutant triggers a significantly accentuated immune response in the *Drosophila* gut as compared to that induced by the wt *Lp*^{WCSF1} strain. Remarkably, the *Diptericin* expression level activated upon the sensing of *Lp*^{WCFS1}*Δdlt* exceeds that of the negative regulators encoding genes (Figure 3C and 3D). The expression of all induced genes is strictly dependent on the activation of the IMD pathway as attested by the phenotype of *relish* or the *PGRP-LC/LE* double mutants (Figure 3B, 3C and 3D). These results contrast with those obtained for the wt *Lp*^{WCFS1} strain. Of note, flies were daily transferred to new vials supplemented by the corresponding bacterial strains and variations in the ingested bacterial loads were excluded by the comparison of the CFU counts in the guts of flies monoassociated with the wt *Lp*^{WCFS1}or the *Lp*^{WCFS1}*Δdlt* mutant strains (Figure 3 – figure supplement 3). Taken together, our data indicate that the D-alanylation of TAs in *L. plantarum* is essential for the immune-modulatory potential of *L. plantarum* and its persistence in the *Drosophila* gut as a prevalent element of its microbiota.

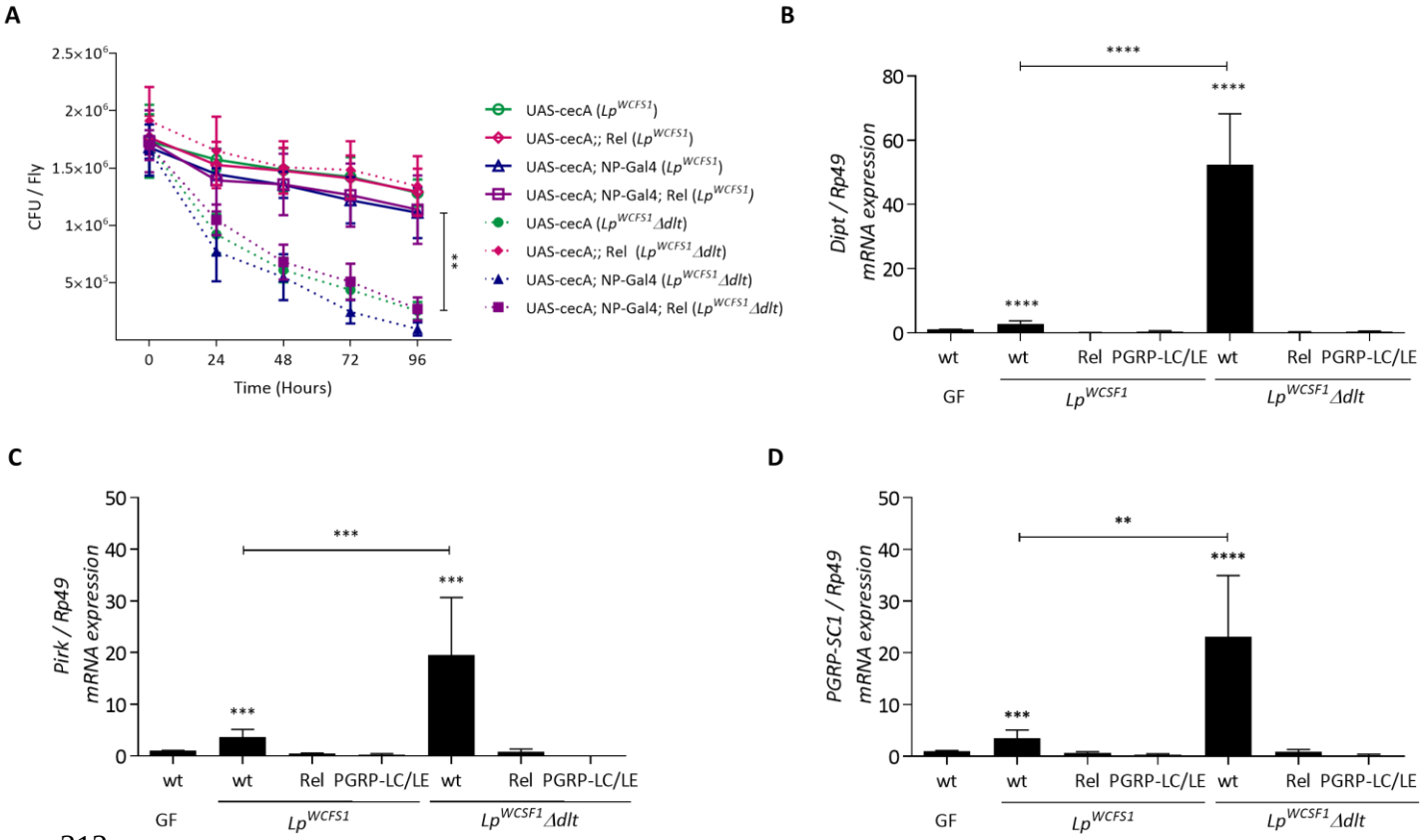

Figure 3: The D-alanylation of TAs is essential for the persistence and the immunomodulatory potential of *Lactobacillus plantarum* in the *Drosophila* gut.

(A) Internal bacterial loads retrieved from the guts of Germ Free (GF) wild-type (wt) or *relish* (Rel) mutant flies fed with Lp^{WCFS1} or $Lp^{WCFS1}\Delta dlt$ for three days. The expression of a *Cecropin* transgene is specifically driven in the gut of these flies by the NP-Gal4 driver. Colony Forming Unit (CFU) counting was performed every 24 hours after transferring the monoassociated flies to a fresh cornmeal medium vial.
(B, C and D) Relative expression of the *Diptericin* (*Dipt*) (B) *Pirk*) (C) and *PGRP-SC1* (D) transcripts in the midgut of Germ Free (GF) wild-type (wt), *relish* (*Rel*) or *PGRP-LC/LE* double mutants flies fed with Lp^{WCFS1} or $Lp^{WCFS1}\Delta dlt$ for 3 days. Transcripts expression was measured by RT-qPCR in total RNA extracts. Ribosomal protein 49 (Rp49) transcript was used as reference gene. Transcripts levels detected in GF flies is set as a control.
Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$). The exact p values are listed in Figure 3 – source data 1.

The following source data and figure supplements are available for figure 3:
Source data 1. P values for figure 3
Figure supplement 1. Lp^{WCFS1} strain shares with Lp^{WJL} strain the ability to colonize the *Drosophila* gut and to induce the IMD pathway.
Figure supplement 2. Overexpression of *Cecropin* A gene in the gut of adult flies.
Figure supplement 3. Internal bacterial loads retrieved from the guts of Germ Free (GF) wild-type flies fed with Lp^{WCFS1} or $Lp^{WCFS1\Delta dlt}$ for three days.
Figure supplement 4. D-alanylation of WTAs are essential for the probiotic effect of Lp^{WCFS1} in *Drosophila*.

335 **D-alanylation of TAs limits the release of PGN fragments through resistance to**
336 **antimicrobial effectors**

337 Our findings so far demonstrate a dual property for D-alanylation of TAs in *Bacilli*: first, this
338 modification of the bacterial surface allows resistance to cationic antimicrobial effectors and
339 second, it hampers sensing by the IMD pathway thus partially eluding the innate immune
340 response. Previous studies have established that monomeric and polymeric fragments of PGN
341 elicit the IMD immune response (Kaneko et al., 2004; Stenbak et al., 2004). Consistently, we
342 reasoned that the *dlt*-dependent resistance to cationic antimicrobial effectors of *Bt407* and *L.*
343 *plantarum* would limit any discharge of cell wall PGN thus only triggering a basal IMD
344 response. To directly examine this possibility, we cultivated wt and *dlt* mutants of *Bt407* and *L.*
345 *plantarum* bacterial strains in culture medium supplemented or not by chicken egg lysozyme. We
346 then compared the IMD- induced response in adult flies injected with the supernatant of each of
347 these microbial cultures. Of note, similarly to AMPs, lysozyme is a cationic antibacterial effector
348 that drastically affects the growth of the *dlt* mutants in contrast to the highly resistant wt strains
349 (data not shown). As shown in Figure 4, *Diptericin* expression triggered by the injection of the
350 supernatants of *Bt407* and Lp^{WCFS1} did not vary whether these cultures were treated or not with
351 lysozyme (Figure 4A and 4B). In contrast, *Diptericin* expression significantly increased in flies
352 injected with the supernatants of *Bt407Δdlt* and *LpWCFS1Δdlt* treated with lysozyme (Figure 4A
353 and 4B). This response is strictly dependent on the activation of the IMD pathway as it is
354 reduced in the *relish* mutant. Remarkably, the supernatants of the *dlt* cultures also elicited a
355 significantly higher *Diptericin* expression as compared to that of wt bacteria. Collectively, our
356 results suggest an increased discharge of immune-stimulatory PGN moieties in the supernatant of
357 *dlt* mutants as compared to that of wt cultures.

358 Next, we asked whether the observed variations in the IMD-elicited response are indeed due to
359 variable concentrations of the PGN fragments and not to any binding constraints related to the
360 covalent bonding of D-alanylated TAs to the PGN fragments. Therefore, we purified cell wall

fractions from the wt and *dlt* mutant bacteria and compared the IMD induced response upon their injection in adult flies. The extraction procedure was performed according to two different protocols in order to conserve or to eliminate TAs from the purified PGN fractions (see Experimental procedures). As shown in Figures 4C and 4D the *Diptericin* expression elicited upon the injection of cell wall fractions in the adult flies remains invariable whether these fractions were extracted from wt or *dlt* mutant strains. Moreover, this expression was not altered whether the TAs were removed from the PGN fractions or not. Interestingly, the elicited immune response also increased in a dose dependent manner, further supporting a direct correlation between the dose of bacterial PGN and the extent of the immune response. Thus, altogether, these data confirm that variations in the IMD-induced response elicited by wt and *dlt* mutants can be linked to varying doses of discharged PGN fragments.

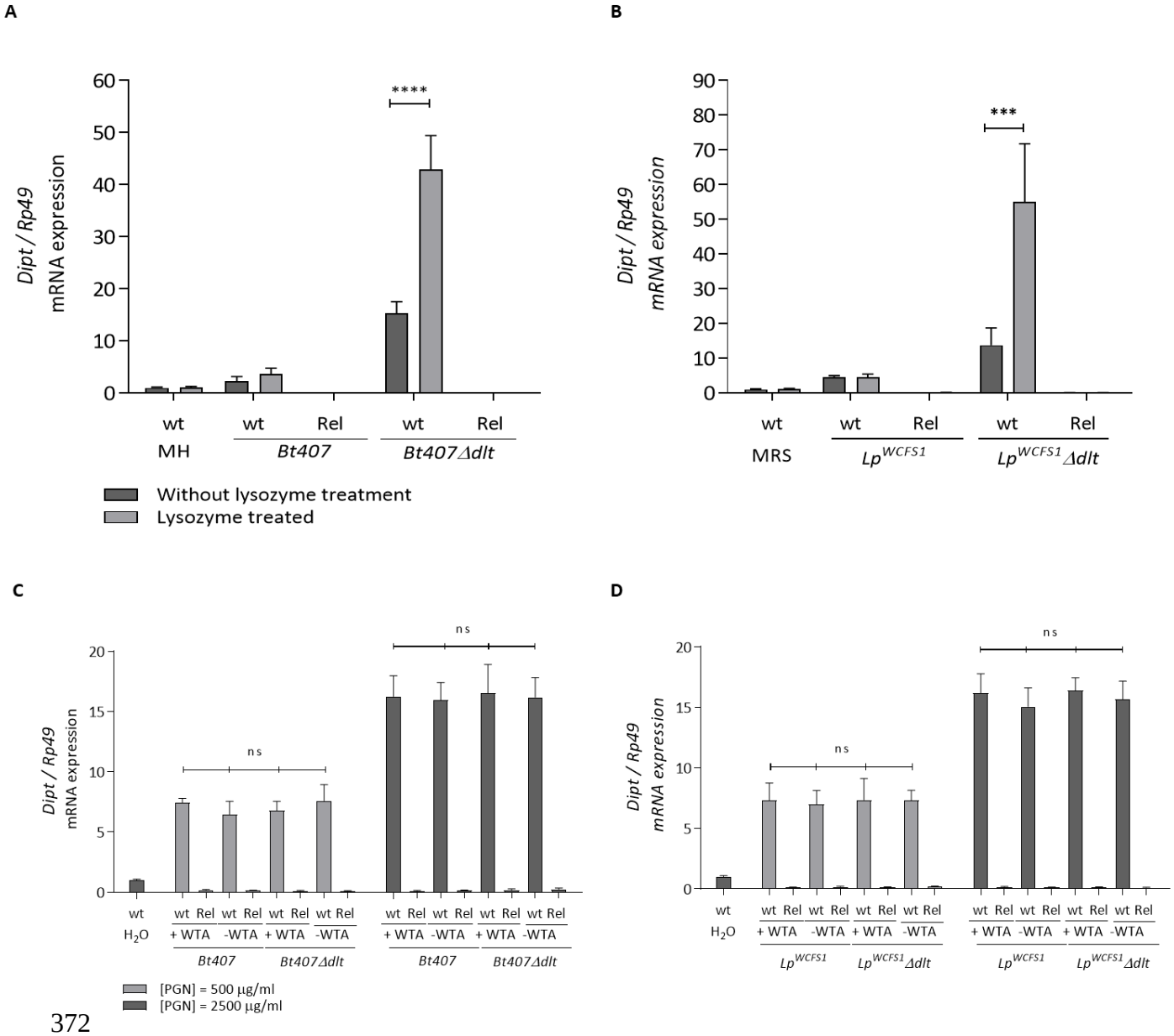

Figure 4: D-alanylation ot TAs limits the release of PGN fragments from the cell wall of *Bacilli* treated by cationic lysozyme.

(A and B) Wild-type (wt) and *relish* (Rel) mutant flies were injected with the supernatant of *Bt407* and Lp^{WCSF1} cultures or their corresponding *dlt* mutants (*Bt407Δdlt* and Lp^{WCSF1}*Δdlt)* treated or not by chicken egg lysozyme (10 mg/ml).
(C and D) Wild-type (wt) and *relish* (Rel) mutant flies were injected with cell wall peptidoglycan (PGN) fractions with or without cell wall TAs (500 and 2500 µg/ml) from the wild type or the *dlt* mutants of*Bt407* and Lp^{WCSF1}.
Relative *Diptericin* (dipt) expression was measured by RT-qPCR 4 h after the microbial induction of the adult flies. Ribosomal protein 49 (Rp49) transcript was used as reference gene. Transcripts levels detected in wt flies injected with MH Broth medium (A), MRS Broth Medium (B) or water (C and D) are set as controls.
Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$). The exact p values are listed in Figure 4 – source data 1.

The following source data is available for figure 4:
Source data 1. p values for figure 4

The *dlt*- dependent resistance of *Lactobacillus plantarum* to lysozyme is essential for modulating the IMD-dependent immune response in the *Drosophila* gut

Our results suggest that the D-alanylation of TAs mitigates the induction of the *Drosophila* IMD pathway by *B. thuringiensis* and *L. plantarum*. More precisely, by providing resistance to cationic antimicrobial effectors, D-alanine esterification of TAs constrains the release of PGN fragments from the bacterial cell wall. To evaluate the relevance of this model, we asked whether the depletion of antimicrobial effectors would alleviate the immune response triggered by the *dlt* mutants *in vivo*. However, as the *Drosophila* genome encodes several AMP genes and that the concomitant inhibition of their expression is quite difficult, we could not address this question in the frame of the systemic immune response triggered by a *B. thuringiensis* septic infection in flies. Therefore, we examined this possibility in the *Drosophila* gut where, in addition to AMPs, lysozyme constitutes another efficient cationic antimicrobial effector. The role of lysozyme in the activation of the IMD-response in the gut has not been considered to date. The *Drosophila* genome comprises seven lysozyme genes of which the members of the LysD family, including *LysD*, *Lys B*, *Lys C* and *Lys E*, and *LysX*, are expressed in the adult gut (Broderick et al., 2014; Daffre et al., 1994). As no lysozyme mutants are available, we expressed a dsRNA targeting the lysozyme transcripts under the control of the gut specific NP-gal4 driver (Hayashi et al., 2002). Since the lysozyme genes share high sequence similarities, the dsRNA constructs targeting the *LysS* and *LysX* transcripts also reduced the expression of all *LysD* like genes as off-targets. We further aggravated this RNAi transcript-depletion by introducing a genetic deficiency uncovering the lysozyme locus into the genome of the transgenic flies (Figure 5 – figure supplement 1). Since we could not establish an oral infection of flies with *B. thuringiensis*, we evaluated the role of gut lysozyme in the modulation of the immune response triggered by the gut resident *L. plantarum*. For that, we quantified *Diptericin* expression level in the guts of GF- RNAi flies recolonized by either the Lp^{WCFS1} or the $Lp^{WCFS1}\Delta dlt$ mutant strains. As shown in figure 5,

compared to control RNAi-GFP flies, the inhibition of lysozyme genes significantly reduced expression of *Diptericin* in flies mono-associated with $Lp^{WCFS1}\Delta dlt$ and the level of reduction in the lysozyme transcripts correlated with the level of reduction in *Diptericin* expression. Of note, *Diptericin* expression did not vary in RNAi treated flies replenished by Lp^{WCFS1} whether lysozyme expression was inhibited or not. These results are in agreement with an increased discharge of PGN fragments from $Lp^{WCFS1}\Delta dlt$ as a result of its vulnerability to cationic antimicrobial effectors *in vivo* and provide a first evidence of the role of lysozyme in modulating the IMD response in the *Drosophila* gut.

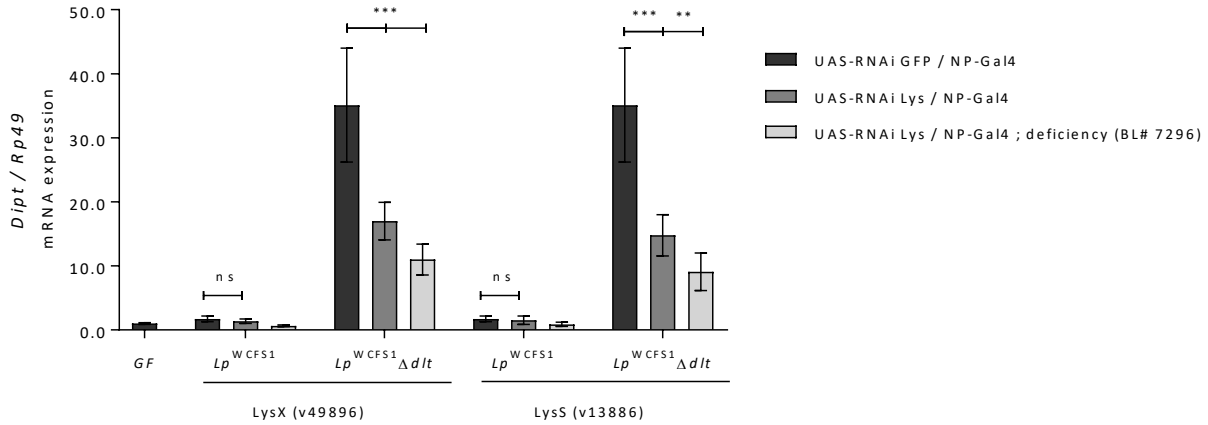

Figure 5: The *dlt*-dependent resistance of *Lactobacillus plantarum* to intestinal lysozyme is essential for the modulation of its ensued IMD- immune response in the gut

Silencing of the lysozyme genes was performed by the expression of RNAi constructs (UAS-RNAi) in the gut of adult flies using the midgut specific driver NP-Gal4. Two different transgenic fly lines (v49896) and (v13886) were used. These harbor different RNAi construct targeting the *LysX* or the *LysS* transcripts respectively. Silencing of *lysozyme* genes is further aggravated by crossing these transgenic flies to a fly line carrying a chromosomal deletion (deficiency BL#7296) uncovering the *lysozyme* locus in the *Drosophila* genome. A transgenic line expressing an RNAi construct targeting the GFP transcript is used as a control. Relative expression of the *Diptericin* transcript is measured in total RNA extracts from the midgut of Germ Free wild-type flies (GF) or the transgenic flies of the different genetic background fed on Lp^{WCFS1} or $Lp^{WCFS1}\Delta dlt$ for 3 days. Ribosomal protein 49 (Rp49) transcript was used as reference gene. Transcripts levels are compared to that detected in in GF flies that is arbitrary set to 1.
Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$). The exact p values are listed in Figure 5 – source data 1.

The following source data and figure supplement are available for figure 5:
Source Data 1. *p* values for figure 5.
Figure supplement 1. RNAi silencing of *lysozyme* genes in the gut.

Discussion

The role of the *dlt*-operon in mediating resistance to cationic antimicrobial effectors *in vitro* has been attested for several Gram-positive bacteria species (Fabretti et al., 2006; Kovacs et al., 2006; Kristian et al., 2005; Perea Velez et al., 2007). This attribute is associated with an attenuated virulence of the *dlt* mutants for several pathogens including *Listeria monocytogenes*, *Bacillus anthracis* and *Staphylococcus aureus* (Abachin et al., 2002; Collins et al., 2002; Fisher et al., 2006; Kristian et al., 2003; Tabuchi et al., 2010). Only for the latter, a role for the *dlt*-operon in hampering immune sensing and the subsequent activation of the Toll pathway in *Drosophila* has been proposed (Tabuchi et al., 2010). The Toll pathway is preferentially triggered upon the sensing of Lys-type PGN by a receptor complex comprising PGRP-SA and GNBP1, a member of the *Drosophila* Glucan-Binding protein family (Gobert et al., 2003; Michel, Reichhart, Hoffmann, & Royet, 2001). However, although the exact mechanism underlying the inhibitory effect of D-alanine esterification of TAs on the Toll pathway activation remains unknown, the results obtained by Kurokawa *et al*. suggest that it entails the covalent bonding of D-alanylated Wall TAs to PGN (Kurokawa et al., 2011). This model is challenged by a report from Atilano *et al.* which indicates that wall TAs limit the access of PGN to the PGRP-SA receptor independently from the activity of the *dlt*-operon (Atilano, Yates, Glittenberg, Filipe, & Ligoxygakis, 2011). We show here that, the presence of TAs, whether D-alanylated or not, does not alter the IMD response upon injection of purified PGN preparations in flies (Figure 4B). Furthermore, the quantification of the IMD response in flies stimulated by injection of supernatants of bacterial cultures treated or not by chicken egg lysozyme, or increasing concentration of PGN preparations, reveals a correlation between the induced response and the doses of injected PGN fragments (Figure 4 A and B). Overall, our data support a model in which the differential immune response following infection of flies by wt or *dlt* mutant bacteria is linked to variations in the quantities of released PGN that are made available for sensing by the

PGRP-LC/ LE receptors upon lysis by AMPs. The differences between our results and those documented for Toll pathway activation might be indicative of different ligand binding / receptor activation mechanisms for the PGRP-LC/ LE and the PGRP-SA/GNBP1 PRR complex.

Resistance to antimicrobial effectors and evasion from immune detection are prominent features of pathogenic bacteria. Interestingly, our data indicate that the D-alanylation of TAs is a common microbial strategy employed by pathogen and commensal alike to elude and resist immune defenses thus acquiring the capacity to develop or persist in appropriate niches. Indeed, our results reveal the essential role of D-alanine esterification in the resistance of *L. plantarum* to the assault of intestinal cationic lysozyme and thus for its persistence as a core component of the *Drosophila* microbiota (Figure 3A). These findings are in agreement with a previous report attesting of the prominence of D-alanine esterification of TAs for the colonization of *L. reuteri* in the mouse intestinal tract (Perea Velez et al., 2007; Walter et al., 2007). The *dlt* operon was also found to be an essential element for the establishment of *L. casei* in the gut of the rabbit ligated ileal loop model (Licandro-Seraut, Scornec, Pedron, Cavin, & Sansonetti, 2014). Our data also point the crucial role of the D-alanylation of TAs in maintaining the immunomodulatory potential of *L. plantarum* in the *Drosophila* gut. This cell wall modification allows the fine-tuning of IMD signaling as to set an essential immune barrier in the gut, which is a prerequisite for the maintenance of a healthy commensal population. Indeed, several lines of evidence have indicated the harmful effects of an exacerbated IMD response in the *Drosophila* gut (Bonnay et al., 2013; Lhocine et al., 2008; Paredes et al., 2011; Ryu et al., 2008). Notably, a dysregulated IMD response has been associated with commensal dysbiosis and epithelial dysplasia (Guo et al., 2014; Ryu et al., 2008). These data further highlight the importance of the dual role of D-alanylation of TAs in the resistance to antimicrobial effectors and the escape of immune detection as defining characteristics of *L. plantarum* as a *bona fide* beneficial *Drosophila* commensal. In other words, resistance to antimicrobial effectors allows the persistence of *L.*

plantarum in the gut and in the habitat of its host. Whereas limiting the discharge of its PGN immunostimulants, permits the set-up of a steady state immune response that would not perturb the equilibrium of gut commensals. Remarkably, according to our results, this immunomodulation capacity of *L. plantarum* largely relies on its *dlt*-dependent resistance to gut lysozyme (Figure 3B, 3C and 3D). Our genetically based data support the long surmised immunomodulatory role of lysozyme (Callewaert & Michiels, 2010; Ragland & Criss, 2017).

Immunomodulation is as an essential property by which lactic acid bacteria exert their biological functions in mammals. It is also the main criteria for the selection of probiotic strains (Bron, Tomita, Mercenier, & Kleerebezem, 2013; I. C. Lee, Tomita, Kleerebezem, & Bron, 2013; Wells, 2011). Notably, the cytokine profiles triggered upon the sensing of these bacteria in the intestinal mucosa determine the subsequent polarization of T cells, and thus the orientation of the ensuing adaptive immune response. Differential cytokine profiles are triggered upon the sensing of appropriate Microbial Associated Molecular Patterns (MAMPs) of *lactobacilli* by cognate PRRs at the intestinal epithelial barrier (Lebeer, Vanderleyden, & De Keersmaecker, 2010). Notably, previous data have suggested that the sensing of lipoteichoic acid (LTA) is mainly performed by the transmembrane TLR2 receptor, whereas sensing of bacterial PGN fragments is preferentially mediated by intracellular NOD receptors (Macho Fernandez, Pot, & Grangette, 2011; Smelt et al., 2013; Travassos et al., 2004). Interestingly, the wt and the *dlt*-mutant of the Lp^{WCFS1} strain triggered a differential cytokine profiles in mammals (Grangette et al., 2005; Smelt et al., 2013). The transcriptional response triggered by the former is marked by the expression of TLR2-dependent pro-inflammatory IL-12 whereas that activated by the latter is characterized by an enhanced expression of anti-inflammatory IL-10 that is mainly NOD-dependent (Grangette et al., 2005; Macho Fernandez, Pot, et al., 2011; Macho Fernandez, Valenti, et al., 2011). Similar results were obtained with a wt and *dlt*-mutant strain of *L. rhamnosus GG* (Claes et al., 2010). Accordingly, the *dlt* mutants alleviated the inflammatory

response in a mouse colitis model (Claes et al., 2010; Grangette et al., 2005). Despite the complexity of the immune response in the mammalian intestinal mucosa, these data are reminiscent of our results reporting a differential immune response triggered by the wt and the $Lp^{WCFS1\Delta dlt}$. Interestingly, a recent study from Bosco-Drayon and colleagues emphasized the prominent role of the intracellular PGRP-LE receptor as a master sensor of small fragments of PGN (tracheal cytotoxin) in the *Drosophila* gut further underlying the similarity between the function of this receptor and that of mammalian NODs (Bosco-Drayon et al., 2012). Given the sensitivity of the *dlt* mutants of Lactobacilli to antimicrobial effectors (Perea Velez et al., 2007; Walter et al., 2007) and the high occurrence of lysozyme and AMPs at the mammalian intestinal barrier, it is tempting to speculate that a similar mechanism might apply for the regulation of PGN-sensing and the modulation of innate immune responses by commensal and probiotic *Lactobacilli* species in *Drosophila* and mammals (Lebeer et al., 2010; Perea Velez et al., 2007).

Several reports have demonstrated the potential of *L. plantarum* to impact *Drosophila* development and physiology (Blum et al., 2013; Bosco-Drayon et al., 2012; Broderick et al., 2014; Erkosar & Leulier, 2014; Guo et al., 2014; Jones et al., 2013; Matos et al., 2017; Ryu et al., 2008; Sharon et al., 2010; Storelli et al., 2011; A. C. Wong et al., 2014). Notably, work from Leulier's laboratory revealed the importance of this bacterium in promoting juvenile growth during chronic undernutrition (Storelli et al., 2011). Interestingly, Matos *et al.* showed that a functional *dlt*-operon is required for this biological function of *L. plantarum* (Matos et al., 2017). Our data give further evidence of the importance of the *dlt*-dependant D-alanylation of TAs for the beneficial impacts of *L. plantarum* on its host biology in particular by tuning the sensing of PGN immunostimulants, thus establishing an *ad hoc* balanced immune response at the intestinal epithelium. Moreover, in *Drosophila* as in mammals, *L. plantarum* was shown to exert protective probiotic effects. Notably, in a recent study, Blum *et al.* provided evidence of a protective role of *L. plantarum* against intestinal pathogens such as *Serratia marcescens*. In this context,

539 complementation of fly food by *L. plantarum* significantly enhanced the capacity of CR and GF
540 flies to survive an infection by *S. marcescens* (Blum et al., 2013). Interestingly, we showed that
541 this probiotic effect of *L. plantarum* feeding in *Drosophila* is strictly dependent on the activity of
542 its *dlt* operon as evidenced by the phenotype of flies fed by the $Lp^{WCFS1\Delta dlt}$ mutant strain (Figure
543 3 – figure supplement 4). These data are in line with a recent comprehensive study performed by
544 Smelt *et al.*, which demonstrated that the majority of the immunomodulatory effects induced by
545 the probiotic Lp^{WCFS1} strain in mice are dependent on the activity of its *dlt*-operon (Smelt et al.,
546 2013).

547 To summarize, our results put forward the pivotal role of the D-alanine esterification of TAs in
548 modulating the sensing of bacterial PGN by the host immune system by preventing antimicrobial
549 effectors mediated bacterial lysis. Modification of bacterial PGN was previously reported as an
550 immune evasion strategy of pathogens to prevent its degradation by lysozyme and thus the
551 release of immunostimulatory fragments (Boneca et al., 2007; Wolfert, Roychowdhury, &
552 Boons, 2007). The function of the *dlt*-operon is unique in the sense that the modification of a
553 cell-wall component, namely TAs, interferes with the sensing of another component, which is
554 PGN, by the host innate immune sensors. Although the *dlt* operon is highly conserved among
555 Gram-positive species, including saprophytic bacteria, such as the harmless and non-pathogenic
556 *B. subtilis*, our results provide a clear evidence that its function is crucial for the success of
557 pathogenic and commensal species in resisting host innate immune defenses. Given the high
558 conservation of the innate immune system and the prevalence of TAs and PGN as central
559 modulators of host defenses, these findings might yield new insights into the pathogenesis and
560 the probiotic effects of bacterial species and potentially orient the development of therapeutic
561 and prophylactic interventions.

Materials and Methods

Bacterial strains and growth conditions

The *dlt* operon mutant of *B. thuringiensis* was generated from the strain *B. thuringiensis* 407 cry- strain (*Bt407*) (Lereclus, Arantes, Chaufaux, & Lecadet, 1989) as described in (Abi Khattar et al., 2009). Briefly, the demethylated pMAD-updlt-aphA3Km-downdlt plasmid (Abi Khattar et al., 2009) was prepared from *Escherichia coli* ET12567 and introduced, by electroporation, into *Bt407*. The *dltXABCD* genes were replaced with the kanamycin (km) resistant cassette by allelic exchange. Clones resistant to Km and susceptible to Erythromycin (Em) were examined by PCR, and the homologous recombination was verified by sequencing.

The *dltA* mutant of *B. subtilis* was constructed in strain 168 as follows: an internal fragment of the *dltA* gene was amplified with primers BSdltA-*Eco*RI (5'-ggaattcctcggccagcagattcagacagttt-3') and BSdltA-BamH1 (5'-acccggatccatcaggcacatttg-3'). The PCR product and the integrative plasmid pDIA5307 (PMID: 8113162) were digested with *Eco*RI and *Bam*HI and ligated together generating pDA5307-BSdltA. The ligated product was digested with *Sma*I to linearize any pDIA5307 plasmid left, transformed into competent cells of strain 168 and selected on chloramphenicol (4 µg/ml). Clones were validated by PCR and 4 independent clones were stored generating strain 168 Δ*dltA*. Clone 1 was used in this work.

Serratia marcescens Db11 (Nehme et al., 2007) and *E. coli* DH5αGFP (Bonnay et al., 2013) are a kind gift from Dr. Dominique Ferrandon and Pr. Jean Marc Reichhart (UPR9022 – CNRS - Université de Strasbourg) and *L. plantarum* WCFS1 (*Lp* ^{WCFS1}) (Kleerebezem et al., 2003) and its *Δdlt* mutant (*Lp* ^{WCFS1}*Δdlt*) (Palumbo et al., 2006) from Pr. Pascal Hols (Unité de Génétique, Institut des Sciences de la Vie, Université catholique de Louvain, B-1348 Louvain-La-Neuve, Belgium). All bacterial strains, except *L. plantarum*, were cultured on Luria-Bertani medium

(LB) at 37°C and 30°C for *B. thuringiensis*. *L. plantarum* was cultured on Man, Rogosa and Sharpe (MRS) medium at 37°C.

Heat killing of bacteria was performed as described (El Chamy, Leclerc, Caldelari, & Reichhart, 2008) Briefly, bacterial solutions followed two steps of 20 minutes of incubation at 95°C separated by 20 minutes of cooling on ice. Killing was verified by plating 100 µl of each bacterial solution on LB agar plates.

For the preparation of the supernatant of bacterial cultures treated with Lysozyme, chicken egg white lysozyme (10mg/ml) was applied to bacterial cultures of *Lp*^{WCFS1}, *Lp* ^{WCFS1}*Δdlt, Bt407* and *Bt407Δdlt* at an optical density of 0.5 at λ = 600 nm (OD_{600} = 0.5) in Muller Hinton (MH) and MRS mediums respectively. When the OD_{600} reached 2, the bacterial cultures were centrifuged, and supernatants were heated at 95°C for 20 minutes before injection in the flies.

Quantification of D-Alanine from TAs and purification of bacterial cell wall fractions

Quantification of D-Ala esterified to TAs was performed by HPLC as described previously (Kamar et al., 2017) , after release by alkaline hydrolysis of whole cells or cell wall fractions.

Cell walls were prepared from *Lp*^{WCFS1}, *Lp* ^{WCFS1}*Δdlt, Bt407* and *Bt407Δdlt* as described (Bernard et al., 2011). SDS, Pronase (2 mg/ml), Trypsin (200 µg/ml), DNase (50 µg/ml) and RNase (50 µg/ml) treatments were applied to obtain cell walls containing peptidoglycan and covalently attached glycopolymers (WTA, polysaccharides). All incubations were performed at pH ≤ 6.0, to keep D-Ala esterified on WTAs. The presence of D-Ala in the purified cell walls was checked by HPLC as described above.

Purified peptidoglycan was obtained after incubation of cell walls in 48% hydrofluoric acid at 4°C for 16 hours, to remove WTA and other polysaccharides attached covalently to peptidoglycan.

608 **_Drosophila melanogaster_ stocks and maintenance**

609 Oregon-R is used as wild-type. The IMD pathway mutants Relish^{E20} (*Rel*), *PGRP-LC*$^{\Delta E12}$
610 (PGRP-LC) and the *PGRP-LC/LE* double mutant combines the *PGRP-LC*$^{\Delta E12}$ and the *PGRP-*
611 *LE*^{E112} mutations were been described (Gottar et al., 2002; Hedengren et al., 1999; Takehana et
612 al., 2004). The UAS-Cecropin A (UAS-Cec) (Reichhart et al., 2002) is a kind gift from Pr. Jean
613 Marc Reichhart (UPR9022 – CNRS - Université de Strasbourg). Flies carrying an UAS-RNAi
614 against *Lysozyme S* (v13386 GD), *Lysozyme X* (v49896 GD) were obtained from the Vienna
615 *Drosophila* RNAi Center (http://stockcenter.vdrc.at/control/main). UAS-RNAi GFP line (397-
616 05) were obtained from the *Drosophila* Genetic Resource Center (Kyoto, Japan; http://
617 www.dgrc.kit.ac.jp/index.html). The deficiency Df(3L)10F125/TM6B,Tb (BL# 7296) and the fat
618 body driver c564-Gal4 (BL# 6982) are from the Bloomington Stock Center. The adult gut tissues
619 driver NP3080- Gal4 (NP-Gal4) was obtained from the *Drosophila* Genetic Resource at the
620 National Institute of Genetics (Shizuoka, Japan). The Gal4 system activation was performed by
621 incubating 2-3 days old flies for six further days at 29 °C. Fly stocks were raised at 25°C on
622 cornmeal-agar medium rich in yeast (7.25%). For antibiotic treatment the medium was
623 supplemented with a mixture of Ampicillin, Kanamycin, Tetracyclin and Erythromycin at 50
624 ug/ml each (final concentration). Adult female flies, aged between 0 to 1 day old, were fed on
625 this mixture for 3 days. Germ free status was checked by plating adult fly gut on MRS and LB
626 agar mediums.

627 **Infections and survival experiments**

628 Survival experiment were performed on a total of ≥ 45 adult females per genotype (15 to 20
629 individuals per each of the three biological replicates). Batches of 15 to 20 female flies, aged
630 between 2 to 4 days old, were pricked with a tungsten needle previously dipped into a bacterial
631 solution prepared from an overnight culture that was washed and diluted in PBS (1x) to a final
632 OD_{600} = 2. The infected flies were incubated at 29 °C and survival were counted were counted

15, 21 and 25 hours post infection. The *Diptericin* expression was measured by RT-qPCR 4h post infection.

Oral infection of flies by *S. marcescens* was performed as described (Nehme et al., 2007). In brief, an overnight bacterial culture was centrifuged and diluted to a final OD_{600} = 1 in a 50mM sucrose solution. The challenged flies were incubated on 29 °C for 54 h and the *Diptericin* expression was quantified in RNA extracts of 5 to 10 dissected guts by RT-qPCR.

9.2nl of sonicated purified peptidoglycan or 69 nl of supernatant treated with lysozyme were injected with into the thorax of batches of 20 to 25 female flies (aged 2 to 4 days old) with a Nanoject apparatus (Drummond, Broomall, PA). These flies were incubated 4 hours at 29 °C then *Diptericin* expression was quantified in RNA extracts by RT-qPCR.

Fly internal bacterial load quantification

The CFU counting was performed on 30 adult females or 15 to 30 midguts per genotype (10 adults or 5 to 10 midguts per each of the three biological replicates) by plating serial dilutions of lysates obtained from infected flies or midguts on LB or MRS mediums containing the appropriate antibiotic to each strain. Each biological sample was analyzed by two technical replicates per experiment.

Monoassociation of germ-free flies

Germ free female flies were transferred to a new food fly medium covered with 150 µl of bacterial solution prepared from an overnight culture of Lp^{WCFS1} or $Lp^{WCFS1}\Delta dlt$ washed and diluted in MRS to a final OD_{600} = 1. *Diptericin*, *pirk*, *PGRP-SC1* expressions were quantified by RT-qPCR from RNA extracts of 5 to 10 dissected guts from flies fed 3 days on *L. plantarum* feeding.

Maintenance of *L. plantarum* in *Drosophila*'s gut

Batches of 20 Germ free flies were fed Lp^{WCFS1} or $Lp^{WCFS1}\Delta dlt$ for 3 days and then transferred to a fresh cornmeal medium vial. Every 24 hours, 5 to 10 guts were dissected and plated on MRS Medium containing the appropriate antibiotic to each strain.

Quantification of gene expression

Total RNA was extracted with TRI Reagent (Sigma – Aldrich) from 15 to 25 adult females or 5 to 10 dissected adult female guts of each genotype per biological replicate. The reverse transcription was performed on 1 µg of RNA by using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific). cDNA was used as template in PCR reaction with three 10 µl technical replicates of each biological sample. qPCR was performed on an iQ5 Real Time PCR detection system (Biorad) using iTaq Universal Syber Green supermix (Biorad). The amount of RNA detected was normalized to that of the house keeping gene *rp49*. Primers for *Diptericin*, *Attacin D*, *pirk*, *PGRP-SC1*, *rp49* and *lysozymes* genes are listed in the supplementary file 1. Relative gene expression levels between control and experimental samples was determined using the ΔΔCT method. Each experimental sample was compared to each wild-type sample.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0b software ($\alpha = 0.05$) and statistical tests used for each data set are indicated in figure legends.

Acknowledgments

We thank Pr. Jean-Marc Reichhart and Dr. Dominique Ferrandon for critical reading of the manuscript. We thank Pr. Pascals Hols for providing the Lp^{WCFS1} strain and its *Δdlt* mutant. This work was funded by the Research Council of the Saint-Joseph University of Beirut (FS73) and the Lebanese National Council of Scientific Research (CNRS-L/ 02-05-16) and has benefited from the support of the CEDRE program (32942VD) and Institut National de la Recherche

679 Agronomique (INRA). Zaynoun Attieh is supported by a PhD fellowship from the CNRS-L and
680 has also benefited from the SAFAR program and an international mobility scholarship from
681 AgroParisTech (Bourse de mobilité Abies). *B. subtilisΔdlt* mutant was constructed in the frame
682 of a European Research Council starting grant (PGNfromSHAPEtoVIR n°202283) awarded to
683 Ivo G. Boneca.

684 Additional information

685 Competing interests

686 The authors declare that no competing interests exist.

687 Figures Supplements

688 **Figure 1**:

689 **Figure supplement 1**. Overexpression of *CecropinA* gene.

690 **Figure supplement 2**. Amounts of D-alanine released from whole cells by alkaline hydrolysis
691 for the wild type and the *dlt* mutant strains of *Bt407*.

692 **Figure supplement 3.** Complementation of the *Bt407ΔdltX* mutant with the *dltX-ORF*
693 (*Bt407ΔdltΩdltX*) completely restores its virulence similarly to the wt *Bt407* strain.

694 **Figure 3:**

695 **Figure supplement 1**. Lp^{WCFS1} strain shares with Lp^{WJL} strain the ability to colonize the
696 *Drosophila* gut and to induce the IMD pathway.

697 **Figure supplement 2.** Overexpression of *Cecropin* A gene in the gut of adult flies.

698 **Figure supplement 3.** Internal bacterial loads retrieved from the guts of Germ Free (GF) wild-
699 type flies fed with Lp^{WCFS1} or $Lp^{WCFS1\Delta dlt}$ for three days.

700 **Figure supplement 4.** D-alanylation of WTAs are essential for the probiotic effect of Lp^{WCFS1} in
701 *Drosophila*.

Figure 5:

Figure supplement 1. RNAi silencing of *lysozyme* genes in the gut.

Sources Data

Figure 1 – source Data 1. p values for figure 1.

Figure 2 – source Data 1. p values for figure 2.

Figure 3 – source Data 1. p values for figure 3.

Figure 4 – source Data 1. p values for figure 4.

Figure 5 – source Data 1. p values for figure 5.

Additional files

Supplementary files

Supplementary file 1. List of primers used for the RT-qPCR reaction.

References

Abachin, E., Poyart, C., Pellegrini, E., Milohanic, E., Fiedler, F., Berche, P., & Trieu-Cuot, P. (2002). Formation of d-alanyl-lipoteichoic acid is required for adhesion and virulence of listeria monocytogenes. *Mol Microbiol, 43*(1), 1-14.

Abi Khattar, Z., Rejasse, A., Destoumieux-Garzon, D., Escoubas, J. M., Sanchis, V., Lereclus, D., . . . Gaudriault, S. (2009). The dlt operon of bacillus cereus is required for resistance to cationic antimicrobial peptides and for virulence in insects. *J Bacteriol, 191*(22), 7063-7073. doi:10.1128/JB.00892-09

Aggarwal, K., Rus, F., Vriesema-Magnuson, C., Erturk-Hasdemir, D., Paquette, N., & Silverman, N. (2008). Rudra interrupts receptor signaling complexes to negatively regulate the imd pathway. *PLoS Pathog, 4*(8), e1000120. doi:10.1371/journal.ppat.1000120

Aggarwal, K., & Silverman, N. (2008). Positive and negative regulation of the drosophila immune response. *BMB Rep, 41*(4), 267-277.

Anaya-Lopez, J. L., Lopez-Meza, J. E., & Ochoa-Zarzosa, A. (2013). Bacterial resistance to cationic antimicrobial peptides. *Crit Rev Microbiol, 39*(2), 180-195. doi:10.3109/1040841X.2012.699025

Atilano, M. L., Yates, J., Glittenberg, M., Filipe, S. R., & Ligoxygakis, P. (2011). Wall teichoic acids of staphylococcus aureus limit recognition by the drosophila peptidoglycan recognition protein-sa to promote pathogenicity. *PLoS Pathog, 7*(12), e1002421. doi:10.1371/journal.ppat.1002421

Bechinger, B., & Gorr, S. U. (2017). Antimicrobial peptides: Mechanisms of action and resistance. *J Dent Res, 96*(3), 254-260. doi:10.1177/0022034516679973

Bernard, E., Rolain, T., Courtin, P., Guillot, A., Langella, P., Hols, P., & Chapot-Chartier, M. P. (2011). Characterization of o-acetylation of n-acetylglucosamine: A novel structural variation of bacterial peptidoglycan. *J Biol Chem, 286*(27), 23950-23958. doi:10.1074/jbc.M111.241414

Bischoff, V., Vignal, C., Duvic, B., Boneca, I. G., Hoffmann, J. A., & Royet, J. (2006). Downregulation of the drosophila immune response by peptidoglycan-recognition proteins sc1 and sc2. *PLoS Pathog, 2*(2), e14.

Blum, J. E., Fischer, C. N., Miles, J., & Handelsman, J. (2013). Frequent replenishment sustains the beneficial microbiome of drosophila melanogaster. *MBio, 4*(6), e00860-00813. doi:10.1128/mBio.00860-13

Boneca, I. G., Dussurget, O., Cabanes, D., Nahori, M. A., Sousa, S., Lecuit, M., . . . Girardin, S. E. (2007). A critical role for peptidoglycan n-deacetylation in listeria evasion from the host innate immune system. *Proc Natl Acad Sci U S A, 104*(3), 997-1002. doi:10.1073/pnas.0609672104

Bonnay, F., Cohen-Berros, E., Hoffmann, M., Kim, S. Y., Boulianne, G. L., Hoffmann, J. A., . . . Reichhart, J. M. (2013). Big bang gene modulates gut immune tolerance in drosophila. *Proc Natl Acad Sci U S A, 110*(8), 2957-2962. doi:1221910110 [pii] 10.1073/pnas.1221910110

Bosco-Drayon, V., Poidevin, M., Boneca, I. G., Narbonne-Reveau, K., Royet, J., & Charroux, B. (2012). Peptidoglycan sensing by the receptor pgrp-le in the drosophila gut induces immune

753 responses to infectious bacteria and tolerance to microbiota. *Cell Host Microbe, 12*(2), 153-165.
754 doi:S1931-3128(12)00228-4 [pii] 10.1016/j.chom.2012.06.002

755 Broderick, N. A., Buchon, N., & Lemaitre, B. (2014). Microbiota-induced changes in drosophila
756 melanogaster host gene expression and gut morphology. *MBio, 5*(3), e01117-01114.
757 doi:10.1128/mBio.01117-14

758 Bron, P. A., Tomita, S., Mercenier, A., & Kleerebezem, M. (2013). Cell surface-associated
759 compounds of probiotic lactobacilli sustain the strain-specificity dogma. *Curr Opin Microbiol,*
760 *16*(3), 262-269. doi:10.1016/j.mib.2013.06.001

761 Brown, S., Santa Maria, J. P., Jr., & Walker, S. (2013). Wall teichoic acids of gram-positive
762 bacteria. *Annu Rev Microbiol, 67*, 313-336. doi:10.1146/annurev-micro-092412-155620

763 Buchon, N., Broderick, N. A., Chakrabarti, S., & Lemaitre, B. (2009). Invasive and indigenous
764 microbiota impact intestinal stem cell activity through multiple pathways in drosophila. *Genes*
765 *Dev, 23*(19), 2333-2344. doi:23/19/2333 [pii] 10.1101/gad.1827009

766 Buchon, N., Broderick, N. A., & Lemaitre, B. (2013). Gut homeostasis in a microbial world:
767 Insights from drosophila melanogaster. *Nat Rev Microbiol, 11*(9), 615-626. doi:nrmicro3074
768 [pii] 10.1038/nrmicro3074

769 Callewaert, L., & Michiels, C. W. (2010). Lysozymes in the animal kingdom. *J Biosci, 35*(1),
770 127-160.

771 Chandler, J. A., Lang, J. M., Bhatnagar, S., Eisen, J. A., & Kopp, A. (2011). Bacterial
772 communities of diverse drosophila species: Ecological context of a host-microbe model system.
773 *PLoS Genet, 7*(9), e1002272. doi:10.1371/journal.pgen.1002272 PGENETICS-D-11-00559 [pii]

774 Chang, C. I., Chelliah, Y., Borek, D., Mengin-Lecreulx, D., & Deisenhofer, J. (2006). Structure
775 of tracheal cytotoxin in complex with a heterodimeric pattern-recognition receptor. *Science,*
776 *311*(5768), 1761-1764.

777 Chang, C. I., Ihara, K., Chelliah, Y., Mengin-Lecreulx, D., Wakatsuki, S., & Deisenhofer, J.
778 (2005). Structure of the ectodomain of drosophila peptidoglycan-recognition protein lca suggests
779 a molecular mechanism for pattern recognition. *Proc Natl Acad Sci U S A, 102*(29), 10279-
780 10284.

781 Choe, K. M., Lee, H., & Anderson, K. V. (2005). Drosophila peptidoglycan recognition protein
782 lc (pgrp-lc) acts as a signal-transducing innate immune receptor. *Proc Natl Acad Sci U S A,*
783 *102*(4), 1122-1126.

784 Choe, K. M., Werner, T., Stoven, S., Hultmark, D., & Anderson, K. V. (2002). Requirement for
785 a peptidoglycan recognition protein (pgrp) in relish activation and antibacterial immune
786 responses in drosophila. *Science, 296*(5566), 359-362. doi:10.1126/science.1070216 1070216
787 [pii]

788 Claes, I. J., Lebeer, S., Shen, C., Verhoeven, T. L., Dilissen, E., De Hertogh, G., . . . De
789 Keersmaecker, S. C. (2010). Impact of lipoteichoic acid modification on the performance of the
790 probiotic lactobacillus rhamnosus gg in experimental colitis. *Clin Exp Immunol, 162*(2), 306-
791 314. doi:10.1111/j.1365-2249.2010.04228.x

792 Collins, L. V., Kristian, S. A., Weidenmaier, C., Faigle, M., Van Kessel, K. P., Van Strijp, J. A.,
793 . . . Peschel, A. (2002). Staphylococcus aureus strains lacking d-alanine modifications of teichoic

794 acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. *J*
795 *Infect Dis, 186*(2), 214-219. doi:10.1086/341454

796 Corby-Harris, V., Pontaroli, A. C., Shimkets, L. J., Bennetzen, J. L., Habel, K. E., & Promislow,
797 D. E. (2007). Geographical distribution and diversity of bacteria associated with natural
798 populations of drosophila melanogaster. *Appl Environ Microbiol, 73*(11), 3470-3479.
799 doi:AEM.02120-06 [pii] 10.1128/AEM.02120-06

800 Cox, C. R., & Gilmore, M. S. (2007). Native microbial colonization of drosophila melanogaster
801 and its use as a model of enterococcus faecalis pathogenesis. *Infect Immun, 75*(4), 1565-1576.
802 doi:IAI.01496-06 [pii] 10.1128/IAI.01496-06

803 Daffre, S., Kylsten, P., Samakovlis, C., & Hultmark, D. (1994). The lysozyme locus in
804 drosophila melanogaster: An expanded gene family adapted for expression in the digestive tract.
805 *Mol Gen Genet, 242*(2), 152-162.

806 Debabov, D. V., Heaton, M. P., Zhang, Q., Stewart, K. D., Lambalot, R. H., & Neuhaus, F. C.
807 (1996). The d-alanyl carrier protein in lactobacillus casei: Cloning, sequencing, and expression
808 of dltc. *J Bacteriol, 178*(13), 3869-3876.

809 Debabov, D. V., Kiriukhin, M. Y., & Neuhaus, F. C. (2000). Biosynthesis of lipoteichoic acid in
810 lactobacillus rhamnosus: Role of dltd in d-alanylation. *J Bacteriol, 182*(10), 2855-2864.

811 Dommett, R., Zilbauer, M., George, J. T., & Bajaj-Elliott, M. (2005). Innate immune defence in
812 the human gastrointestinal tract. *Mol Immunol, 42*(8), 903-912.
813 doi:10.1016/j.molimm.2004.12.004

814 Dushay, M. S., Asling, B., & Hultmark, D. (1996). Origins of immunity: *Relish*, a compound rel-
815 like gene in the antibacterial defense of *drosophila*. *Proc. Natl. Acad. Sci. (USA), 93*(19), 10343-
816 10347.

817 El Chamy, L., Leclerc, V., Caldelari, I., & Reichhart, J. M. (2008). Sensing of 'danger signals'
818 and pathogen-associated molecular patterns defines binary signaling pathways 'upstream' of toll.
819 *Nat Immunol, 9*(10), 1165-1170. doi:10.1038/ni.1643

820 El Chamy, L., Matt, N., Ntwasa, M., & Reichhart, J. M. (2015). The multilayered innate immune
821 defense of the gut. *Biomed J, 38*(4), 276-284. doi:10.4103/2319-4170.158621

822 Erkosar, B., & Leulier, F. (2014). Transient adult microbiota, gut homeostasis and longevity:
823 Novel insights from the drosophila model. *FEBS Lett, 588*(22), 4250-4257.
824 doi:10.1016/j.febslet.2014.06.041

825 Fabretti, F., Theilacker, C., Baldassarri, L., Kaczynski, Z., Kropec, A., Holst, O., & Huebner, J.
826 (2006). Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and
827 resistance to antimicrobial peptides. *Infect Immun, 74*(7), 4164-4171. doi:10.1128/IAI.00111-06

828 Ferrandon, D. (2013). The complementary facets of epithelial host defenses in the genetic model
829 organism drosophila melanogaster: From resistance to resilience. *Curr Opin Immunol, 25*(1), 59-
830 70. doi:10.1016/j.coi.2012.11.008

831 Ferrandon, D., Imler, J. L., Hetru, C., & Hoffmann, J. A. (2007). The drosophila systemic
832 immune response: Sensing and signalling during bacterial and fungal infections. *Nat Rev*
833 *Immunol, 7*(11), 862-874. doi:10.1038/nri2194

834 Fisher, N., Shetron-Rama, L., Herring-Palmer, A., Heffernan, B., Bergman, N., & Hanna, P.
835 (2006). The dltabcd operon of bacillus anthracis sterne is required for virulence and resistance to
836 peptide, enzymatic, and cellular mediators of innate immunity. *J Bacteriol, 188*(4), 1301-1309.
837 doi:10.1128/JB.188.4.1301-1309.2006

838 Gobert, V., Gottar, M., Matskevich, A. A., Rutschmann, S., Royet, J., Belvin, M., . . . Ferrandon,
839 D. (2003). Dual activation of the drosophila toll pathway by two pattern recognition receptors.
840 *Science, 302*(5653), 2126-2130. doi:10.1126/science.1085432 302/5653/2126 [pii]

841 Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J. A., . . . Royet, J. (2002).
842 The drosophila immune response against gram-negative bacteria is mediated by a peptidoglycan
843 recognition protein. *Nature, 416*(6881), 640-644. doi:10.1038/nature734 nature734 [pii]

844 Grangette, C., Nutten, S., Palumbo, E., Morath, S., Hermann, C., Dewulf, J., . . . Mercenier, A.
845 (2005). Enhanced antiinflammatory capacity of a lactobacillus plantarum mutant synthesizing
846 modified teichoic acids. *Proc Natl Acad Sci U S A, 102*(29), 10321-10326.
847 doi:10.1073/pnas.0504084102

848 Guo, Y., Li, Z., & Lin, X. (2014). Hs3st-a and hs3st-b regulate intestinal homeostasis in
849 drosophila adult midgut. *Cell Signal, 26*(11), 2317-2325. doi:10.1016/j.cellsig.2014.07.015

850 Harrison, D. A., Binari, R., Nahreini, T. S., Gilman, M., & Perrimon, N. (1995). Activation of a
851 drosophila janus kinase (jak) causes hematopoietic neoplasia and developmental defects. *EMBO*
852 *J, 14*(12), 2857-2865.

853 Hayashi, S., Ito, K., Sado, Y., Taniguchi, M., Akimoto, A., Takeuchi, H., . . . Goto, S. (2002).
854 Getdb, a database compiling expression patterns and molecular locations of a collection of gal4
855 enhancer traps. *Genesis, 34*(1-2), 58-61. doi:10.1002/gene.10137

856 Hayward, A., & Davis, G. (1956). The isolation and classification of lactobacillus strains from
857 italian saliva samples. *Br. Dent. J, 101*, 2733-2741.

858 Heaton, M. P., & Neuhaus, F. C. (1992). Biosynthesis of d-alanyl-lipoteichoic acid: Cloning,
859 nucleotide sequence, and expression of the lactobacillus casei gene for the d-alanine-activating
860 enzyme. *J Bacteriol, 174*(14), 4707-4717.

861 Heaton, M. P., & Neuhaus, F. C. (1994). Role of the d-alanyl carrier protein in the biosynthesis
862 of d-alanyl-lipoteichoic acid. *J Bacteriol, 176*(3), 681-690.

863 Hedengren, M., Asling, B., Dushay, M. S., Ando, I., Ekengren, S., Wihlborg, M., & Hultmark,
864 D. (1999). *Relish*, a central factor in the control of humoral but not cellular immunity in
865 *drosophila*. *Molecular Cell, 4*, 1-20.

866 Hold, G. L., Mukhopadhya, I., & Monie, T. P. (2011). Innate immune sensors and
867 gastrointestinal bacterial infections. *Clin Dev Immunol, 2011*, 579650. doi:10.1155/2011/579650

868 Iatsenko, I., Boquete, J. P., & Lemaitre, B. (2018). Microbiota-derived lactate activates
869 production of reactive oxygen species by the intestinal nadph oxidase nox and shortens
870 drosophila lifespan. *Immunity, 49*(5), 929-942 e925. doi:10.1016/j.immuni.2018.09.017

871 Imler, J. L., & Bulet, P. (2005). Antimicrobial peptides in drosophila: Structures, activities and
872 gene regulation. *Chem Immunol Allergy, 86*, 1-21. doi:10.1159/000086648

873 Jones, R. M., Luo, L., Ardita, C. S., Richardson, A. N., Kwon, Y. M., Mercante, J. W., . . .
874 Neish, A. S. (2013). Symbiotic lactobacilli stimulate gut epithelial proliferation via nox-mediated
875 generation of reactive oxygen species. *EMBO J, 32*(23), 3017-3028.
876 doi:10.1038/emboj.2013.224

877 Joo, H. S., Fu, C. I., & Otto, M. (2016). Bacterial strategies of resistance to antimicrobial
878 peptides. *Philos Trans R Soc Lond B Biol Sci, 371*(1695). doi:10.1098/rstb.2015.0292

879 Kamar, R., Rejasse, A., Jehanno, I., Attieh, Z., Courtin, P., Chapot-Chartier, M. P., . . . Sanchis-
880 Borja, V. (2017). Dltx of bacillus thuringiensis is essential for d-alanylation of teichoic acids and
881 resistance to antimicrobial response in insects. *Front Microbiol, 8*, 1437.
882 doi:10.3389/fmicb.2017.01437

883 Kaneko, T., Goldman, W. E., Mellroth, P., Steiner, H., Fukase, K., Kusumoto, S., . . . Silverman,
884 N. (2004). Monomeric and polymeric gram-negative peptidoglycan but not purified lps stimulate
885 the drosophila imd pathway. *Immunity, 20*(5), 637-649. doi:S1074761304001049 [pii]

886 Kaneko, T., Yano, T., Aggarwal, K., Lim, J. H., Ueda, K., Oshima, Y., . . . Silverman, N. (2006).
887 Pgrp-lc and pgrp-le have essential yet distinct functions in the drosophila immune response to
888 monomeric dap-type peptidoglycan. *Nat Immunol, 7*(7), 715-723. doi:ni1356 [pii]
889 10.1038/ni1356

890 Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O. P., Leer, R., . . .
891 Siezen, R. J. (2003). Complete genome sequence of lactobacillus plantarum wcfs1. *Proc Natl*
892 *Acad Sci U S A, 100*(4), 1990-1995. doi:10.1073/pnas.0337704100

893 Kleerebezem, M., Hols, P., Bernard, E., Rolain, T., Zhou, M., Siezen, R. J., & Bron, P. A.
894 (2010). The extracellular biology of the lactobacilli. *FEMS Microbiol Rev, 34*(2), 199-230.
895 doi:10.1111/j.1574-6976.2010.00208.x

896 Kleino, A., Myllymaki, H., Kallio, J., Vanha-aho, L. M., Oksanen, K., Ulvila, J., . . . Ramet, M.
897 (2008). Pirk is a negative regulator of the drosophila imd pathway. *J Immunol, 180*(8), 5413-
898 5422. doi:180/8/5413 [pii]

899 Kleino, A., Ramia, N. F., Bozkurt, G., Shen, Y., Nailwal, H., Huang, J., . . . Silverman, N.
900 (2017). Peptidoglycan-sensing receptors trigger the formation of functional amyloids of the
901 adaptor protein imd to initiate drosophila nf-kappab signaling. *Immunity, 47*(4), 635-647 e636.
902 doi:10.1016/j.immuni.2017.09.011

903 Koprivnjak, T., & Peschel, A. (2011). Bacterial resistance mechanisms against host defense
904 peptides. *Cell Mol Life Sci, 68*(13), 2243-2254. doi:10.1007/s00018-011-0716-4

905 Kovacs, M., Halfmann, A., Fedtke, I., Heintz, M., Peschel, A., Vollmer, W., . . . Bruckner, R.
906 (2006). A functional dlt operon, encoding proteins required for incorporation of d-alanine in
907 teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in
908 streptococcus pneumoniae. *J Bacteriol, 188*(16), 5797-5805. doi:10.1128/JB.00336-06

909 Kristian, S. A., Datta, V., Weidenmaier, C., Kansal, R., Fedtke, I., Peschel, A., . . . Nizet, V.
910 (2005). D-alanylation of teichoic acids promotes group a streptococcus antimicrobial peptide
911 resistance, neutrophil survival, and epithelial cell invasion. *J Bacteriol, 187*(19), 6719-6725.
912 doi:10.1128/JB.187.19.6719-6725.2005

913 Kristian, S. A., Lauth, X., Nizet, V., Goetz, F., Neumeister, B., Peschel, A., & Landmann, R.
914 (2003). Alanylation of teichoic acids protects staphylococcus aureus against toll-like receptor 2-
915 dependent host defense in a mouse tissue cage infection model. *J Infect Dis, 188*(3), 414-423.
916 doi:10.1086/376533

917 Kurokawa, K., Gong, J. H., Ryu, K. H., Zheng, L., Chae, J. H., Kim, M. S., & Lee, B. L. (2011).
918 Biochemical characterization of evasion from peptidoglycan recognition by staphylococcus
919 aureus d-alanylated wall teichoic acid in insect innate immunity. *Dev Comp Immunol, 35*(8),
920 835-839. doi:10.1016/j.dci.2011.03.001

921 Lebeer, S., Vanderleyden, J., & De Keersmaecker, S. C. (2010). Adaptation factors of the
922 probiotic lactobacillus rhamnosus gg. *Benef Microbes, 1*(4), 335-342. doi:10.3920/BM2010.0032

923 Lee, I. C., Tomita, S., Kleerebezem, M., & Bron, P. A. (2013). The quest for probiotic effector
924 molecules--unraveling strain specificity at the molecular level. *Pharmacol Res, 69*(1), 61-74.
925 doi:10.1016/j.phrs.2012.09.010

926 Lee, W. J., & Brey, P. T. (2013). How microbiomes influence metazoan development: Insights
927 from history and drosophila modeling of gut-microbe interactions. *Annu Rev Cell Dev Biol, 29*,
928 571-592. doi:10.1146/annurev-cellbio-101512-122333

929 Lemaitre, B., & Hoffmann, J. (2007). The host defense of drosophila melanogaster. *Annu Rev*
930 *Immunol, 25*, 697-743. doi:10.1146/annurev.immunol.25.022106.141615

931 Lereclus, D., Arantes, O., Chaufaux, J., & Lecadet, M. (1989). Transformation and expression of
932 a cloned delta-endotoxin gene in bacillus thuringiensis. *FEMS Microbiol Lett, 51*(1), 211-217.

933 Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J. H., Caroff, M., Lee, W. J., . . . Lemaitre, B.
934 (2003). The drosophila immune system detects bacteria through specific peptidoglycan
935 recognition. *Nat Immunol, 4*(5), 478-484. doi:10.1038/ni922 ni922 [pii]

936 Lhocine, N., Ribeiro, P. S., Buchon, N., Wepf, A., Wilson, R., Tenev, T., . . . Leulier, F. (2008).
937 Pims modulates immune tolerance by negatively regulating drosophila innate immune signaling.
938 *Cell Host Microbe, 4*(2), 147-158. doi:S1931-3128(08)00222-9 [pii]
939 10.1016/j.chom.2008.07.004

940 Licandro-Seraut, H., Scornec, H., Pedron, T., Cavin, J. F., & Sansonetti, P. J. (2014). Functional
941 genomics of lactobacillus casei establishment in the gut. *Proc Natl Acad Sci U S A, 111*(30),
942 E3101-3109. doi:10.1073/pnas.1411883111

943 Ma, D., Storelli, G., Mitchell, M., & Leulier, F. (2015). Studying host-microbiota mutualism in
944 drosophila: Harnessing the power of gnotobiotic flies. *Biomed J, 38*(4), 285-293.
945 doi:10.4103/2319-4170.158620

946 Macho Fernandez, E., Pot, B., & Grangette, C. (2011). Beneficial effect of probiotics in ibd: Are
947 peptidogycan and nod2 the molecular key effectors? *Gut Microbes, 2*(5), 280-286.
948 doi:10.4161/gmic.2.5.18255

949 Macho Fernandez, E., Valenti, V., Rockel, C., Hermann, C., Pot, B., Boneca, I. G., & Grangette,
950 C. (2011). Anti-inflammatory capacity of selected lactobacilli in experimental colitis is driven by
951 nod2-mediated recognition of a specific peptidoglycan-derived muropeptide. *Gut, 60*(8), 1050-
952 1059. doi:10.1136/gut.2010.232918

Matos, R. C., Schwarzer, M., Gervais, H., Courtin, P., Joncour, P., Gillet, B., . . . Leulier, F. (2017). D-alanylation of teichoic acids contributes to lactobacillus plantarum-mediated drosophila growth during chronic undernutrition. *Nat Microbiol, 2*(12), 1635-1647. doi:10.1038/s41564-017-0038-x

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. *Nature, 449*(7164), 819-826. doi:10.1038/nature06246

Mellroth, P., Karlsson, J., & Steiner, H. (2003). A scavenger function for a drosophila peptidoglycan recognition protein. *J Biol Chem, 278*(9), 7059-7064.

Mellroth, P., & Steiner, H. (2006). Pgrp-sb1: An n-acetylmuramoyl l-alanine amidase with antibacterial activity. *Biochem Biophys Res Commun, 350*(4), 994-999.

Michel, T., Reichhart, J. M., Hoffmann, J. A., & Royet, J. (2001). Drosophila toll is activated by gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature, 414*(6865), 756-759. doi:10.1038/414756a 414756a [pii]

Nehme, N. T., Liegeois, S., Kele, B., Giammarinaro, P., Pradel, E., Hoffmann, J. A., . . . Ferrandon, D. (2007). A model of bacterial intestinal infections in drosophila melanogaster. *PLoS Pathog, 3*(11), e173. doi:07-PLPA-RA-0055 [pii] 10.1371/journal.ppat.0030173

Neuhaus, F. C., & Baddiley, J. (2003). A continuum of anionic charge: Structures and functions of d-alanyl-teichoic acids in gram-positive bacteria. *Microbiol Mol Biol Rev, 67*(4), 686-723.

Newell, P. D., Chaston, J. M., Wang, Y., Winans, N. J., Sannino, D. R., Wong, A. C., . . . Douglas, A. E. (2014). In vivo function and comparative genomic analyses of the drosophila gut microbiota identify candidate symbiosis factors. *Front Microbiol, 5*, 576. doi:10.3389/fmicb.2014.00576

Palumbo, E., Deghorain, M., Cocconcelli, P. S., Kleerebezem, M., Geyer, A., Hartung, T., . . . Hols, P. (2006). D-alanyl ester depletion of teichoic acids in lactobacillus plantarum results in a major modification of lipoteichoic acid composition and cell wall perforations at the septum mediated by the acm2 autolysin. *J Bacteriol, 188*(10), 3709-3715. doi:10.1128/JB.188.10.3709-3715.2006

Paredes, J. C., Welchman, D. P., Poidevin, M., & Lemaitre, B. (2011). Negative regulation by amidase pgrps shapes the drosophila antibacterial response and protects the fly from innocuous infection. *Immunity, 35*(5), 770-779. doi:S1074-7613(11)00466-3 [pii] 10.1016/j.immuni.2011.09.018

Perea Velez, M., Verhoeven, T. L., Draing, C., Von Aulock, S., Pfitzenmaier, M., Geyer, A., . . . De Keersmaecker, S. C. (2007). Functional analysis of d-alanylation of lipoteichoic acid in the probiotic strain lactobacillus rhamnosus gg. *Appl Environ Microbiol, 73*(11), 3595-3604. doi:10.1128/AEM.02083-06

Perego, M., Glaser, P., Minutello, A., Strauch, M. A., Leopold, K., & Fischer, W. (1995). Incorporation of d-alanine into lipoteichoic acid and wall teichoic acid in bacillus subtilis. Identification of genes and regulation. *J Biol Chem, 270*(26), 15598-15606.

Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G., & Gotz, F. (1999). Inactivation of the dlt operon in staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J Biol Chem, 274*(13), 8405-8410.

994 Poyart, C., Pellegrini, E., Marceau, M., Baptista, M., Jaubert, F., Lamy, M. C., & Trieu-Cuot, P.
995 (2003). Attenuated virulence of streptococcus agalactiae deficient in d-alanyl-lipoteichoic acid is
996 due to an increased susceptibility to defensins and phagocytic cells. *Mol Microbiol, 49*(6), 1615-
997 1625.

998 Ragland, S. A., & Criss, A. K. (2017). From bacterial killing to immune modulation: Recent
999 insights into the functions of lysozyme. *PLoS Pathog, 13*(9), e1006512.
1000 doi:10.1371/journal.ppat.1006512

1001 Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., & Ezekowitz, R. A. (2002).
1002 Functional genomic analysis of phagocytosis and identification of a drosophila receptor for e.
1003 Coli. *Nature, 416*(6881), 644-648. doi:10.1038/nature735 nature735 [pii]

1004 Reichhart, J. M., Ligoxygakis, P., Naitza, S., Woerfel, G., Imler, J. L., & Gubb, D. (2002).
1005 Splice-activated uas hairpin vector gives complete rnai knockout of single or double target
1006 transcripts in drosophila melanogaster. *Genesis, 34*(1-2), 160-164.

1007 Ryu, J. H., Kim, S. H., Lee, H. Y., Bai, J. Y., Nam, Y. D., Bae, J. W., . . . Lee, W. J. (2008).
1008 Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in
1009 drosophila. *Science, 319*(5864), 777-782. doi:1149357 [pii] 10.1126/science.1149357

1010 Ryu, J. H., Nam, K. B., Oh, C. T., Nam, H. J., Kim, S. H., Yoon, J. H., . . . Lee, W. J. (2004).
1011 The homeobox gene caudal regulates constitutive local expression of antimicrobial peptide genes
1012 in drosophila epithelia. *Mol Cell Biol, 24*(1), 172-185.

1013 Sharon, G., Segal, D., Ringo, J. M., Hefetz, A., Zilber-Rosenberg, I., & Rosenberg, E. (2010).
1014 Commensal bacteria play a role in mating preference of drosophila melanogaster. *Proc Natl*
1015 *Acad Sci U S A, 107*(46), 20051-20056. doi:1009906107 [pii] 10.1073/pnas.1009906107

1016 Shin, S. C., Kim, S. H., You, H., Kim, B., Kim, A. C., Lee, K. A., . . . Lee, W. J. (2011).
1017 Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin
1018 signaling. *Science, 334*(6056), 670-674. doi:10.1126/science.1212782

1019 Smelt, M. J., de Haan, B. J., Bron, P. A., van Swam, I., Meijerink, M., Wells, J. M., . . . de Vos,
1020 P. (2013). The impact of lactobacillus plantarum wcfs1 teichoic acid d-alanylation on the
1021 generation of effector and regulatory t-cells in healthy mice. *PLoS One, 8*(4), e63099.
1022 doi:10.1371/journal.pone.0063099

1023 Staubach, F., Baines, J. F., Kunzel, S., Bik, E. M., & Petrov, D. A. (2013). Host species and
1024 environmental effects on bacterial communities associated with drosophila in the laboratory and
1025 in the natural environment. *PLoS One, 8*(8), e70749. doi:10.1371/journal.pone.0070749

1026 Stein, D., Roth, S., Vogelsang, E., & Nüsslein-Volhard, C. (1991). The polarity of the
1027 dorsoventral axis of the drosophila embryo is defined by an extracellular signal. *Cell, 65*, 725-
1028 735.

1029 Stenbak, C. R., Ryu, J. H., Leulier, F., Pili-Floury, S., Parquet, C., Herve, M., . . . Mengin-
1030 Lecreulx, D. (2004). Peptidoglycan molecular requirements allowing detection by the drosophila
1031 immune deficiency pathway. *J Immunol, 173*(12), 7339-7348. doi:173/12/7339 [pii]

1032 Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J., & Leulier, F. (2011). Lactobacillus
1033 plantarum promotes drosophila systemic growth by modulating hormonal signals through tor-

dependent nutrient sensing. *Cell Metab, 14*(3), 403-414. doi:S1550-4131(11)00306-8 [pii] 10.1016/j.cmet.2011.07.012

Tabuchi, Y., Shiratsuchi, A., Kurokawa, K., Gong, J. H., Sekimizu, K., Lee, B. L., & Nakanishi, Y. (2010). Inhibitory role for d-alanylation of wall teichoic acid in activation of insect toll pathway by peptidoglycan of staphylococcus aureus. *J Immunol, 185*(4), 2424-2431. doi:10.4049/jimmunol.1000625

Takehana, A., Yano, T., Mita, S., Kotani, A., Oshima, Y., & Kurata, S. (2004). Peptidoglycan recognition protein (pgrp)-le and pgrp-lc act synergistically in drosophila immunity. *EMBO J, 23*(23), 4690-4700. doi:7600466 [pii] 10.1038/sj.emboj.7600466

Travassos, L. H., Girardin, S. E., Philpott, D. J., Blanot, D., Nahori, M. A., Werts, C., & Boneca, I. G. (2004). Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Rep, 5*(10), 1000-1006. doi:10.1038/sj.embor.7400248

Walter, J., Loach, D. M., Alqumber, M., Rockel, C., Hermann, C., Pfitzenmaier, M., & Tannock, G. W. (2007). D-alanyl ester depletion of teichoic acids in lactobacillus reuteri 100-23 results in impaired colonization of the mouse gastrointestinal tract. *Environ Microbiol, 9*(7), 1750-1760. doi:10.1111/j.1462-2920.2007.01292.x

Wells, J. M. (2011). Immunomodulatory mechanisms of lactobacilli. *Microb Cell Fact, 10 Suppl 1*, S17. doi:10.1186/1475-2859-10-S1-S17

Wolfert, M. A., Roychowdhury, A., & Boons, G. J. (2007). Modification of the structure of peptidoglycan is a strategy to avoid detection by nucleotide-binding oligomerization domain protein 1. *Infect Immun, 75*(2), 706-713. doi:10.1128/IAI.01597-06

Wong, A. C., Dobson, A. J., & Douglas, A. E. (2014). Gut microbiota dictates the metabolic response of drosophila to diet. *J Exp Biol, 217*(Pt 11), 1894-1901. doi:10.1242/jeb.101725

Wong, C. N., Ng, P., & Douglas, A. E. (2011). Low-diversity bacterial community in the gut of the fruitfly drosophila melanogaster. *Environ Microbiol, 13*(7), 1889-1900. doi:10.1111/j.1462-2920.2011.02511.x

Zaidman-Remy, A., Herve, M., Poidevin, M., Pili-Floury, S., Kim, M. S., Blanot, D., . . . Lemaitre, B. (2006). The drosophila amidase pgrp-lb modulates the immune response to bacterial infection. *Immunity, 24*(4), 463-473. doi:S1074-7613(06)00177-4 [pii] 10.1016/j.immuni.2006.02.012

Figures supplements

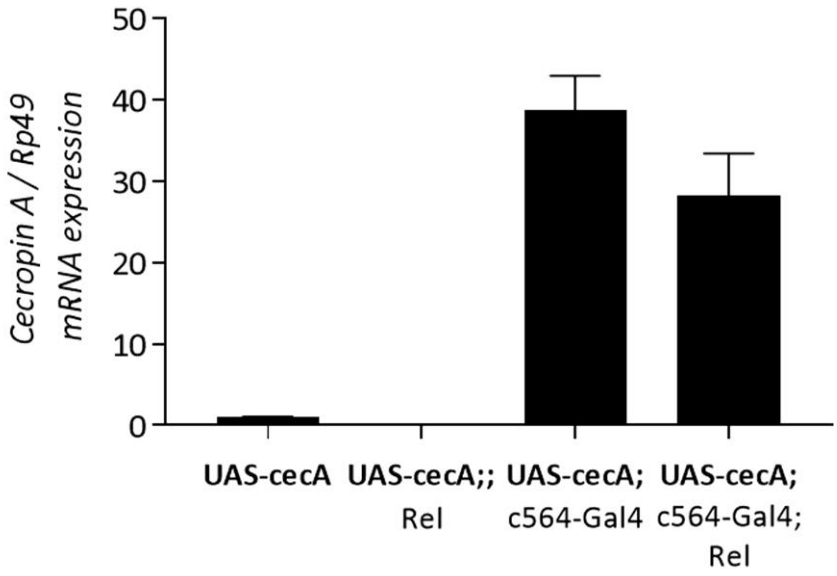

Figure 1 – figure supplement 1: Overexpression of *CecropinA* gene.

The expression of a *Cecropin A* transgene (UAS-cec) was driven using a fat body driver, c564-Gal4, in wild-type (wt) or *Rel* mutant flies. The *Cecropin A* expression a was quantified by RT-qPCR from the RNA extracts of adult flies following their incubation for 6 days at 29 °C. Wt flies carrying the UAS- cec transgene were used as control. Data obtained from three independent experiments are combined in single value (mean ± sd).

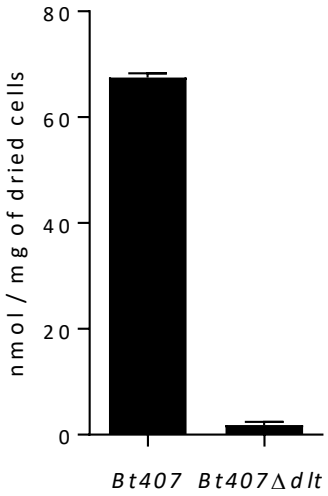

Figure 1 – figure supplement 2: Amounts of D-alanine released from whole cells by alkaline hydrolysis for the wild type and the *dlt* mutant strains of *Bt407*.

Data obtained from three independent experiments are combined in single value (mean ± sd).

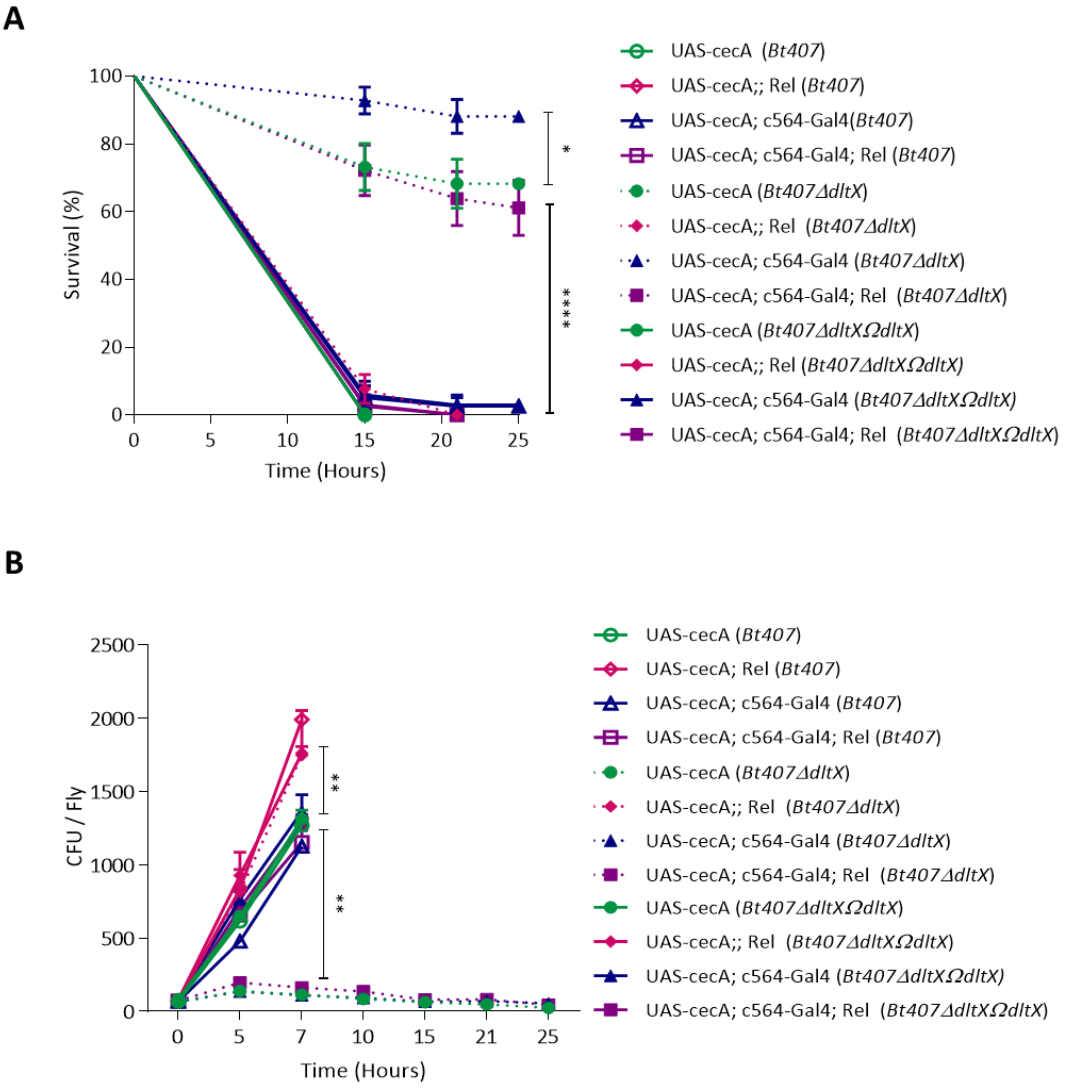

Figure 1 – figure supplement 3: Complementation of the *Bt407ΔdltX* mutant with the *dltX-ORF* (*Bt407ΔdltXΩdltX*) completely restores its virulence similarly to the wt *Bt407* strain.

(A) Survival of adult wild-type (wt) or relish (Rel) mutant, overexpressing or not a *Cecropin* transgene (under the control of the c564-Gal4 fat body driver) to an infection with *Bt407, Bt407ΔdltX and Bt407ΔdltX ΩdltX*
(B) Internal bacterial loads retrieved from adult flies infected with *BT407*and *Bt407ΔdltX* or *Bt407ΔdltX ΩdltX*. The Colony Forming Unit (CFU) counting was performed only on survival flies.
Data are pooled from three independent experiments (mean and s.d.). Statistical tests were performed using the Log Rank test for the survival assays and Mann-Whitney test for the CFU counting within Prism software (ns: $p >$ 0.05; * $0.01 < p < 0.05$; **: $0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

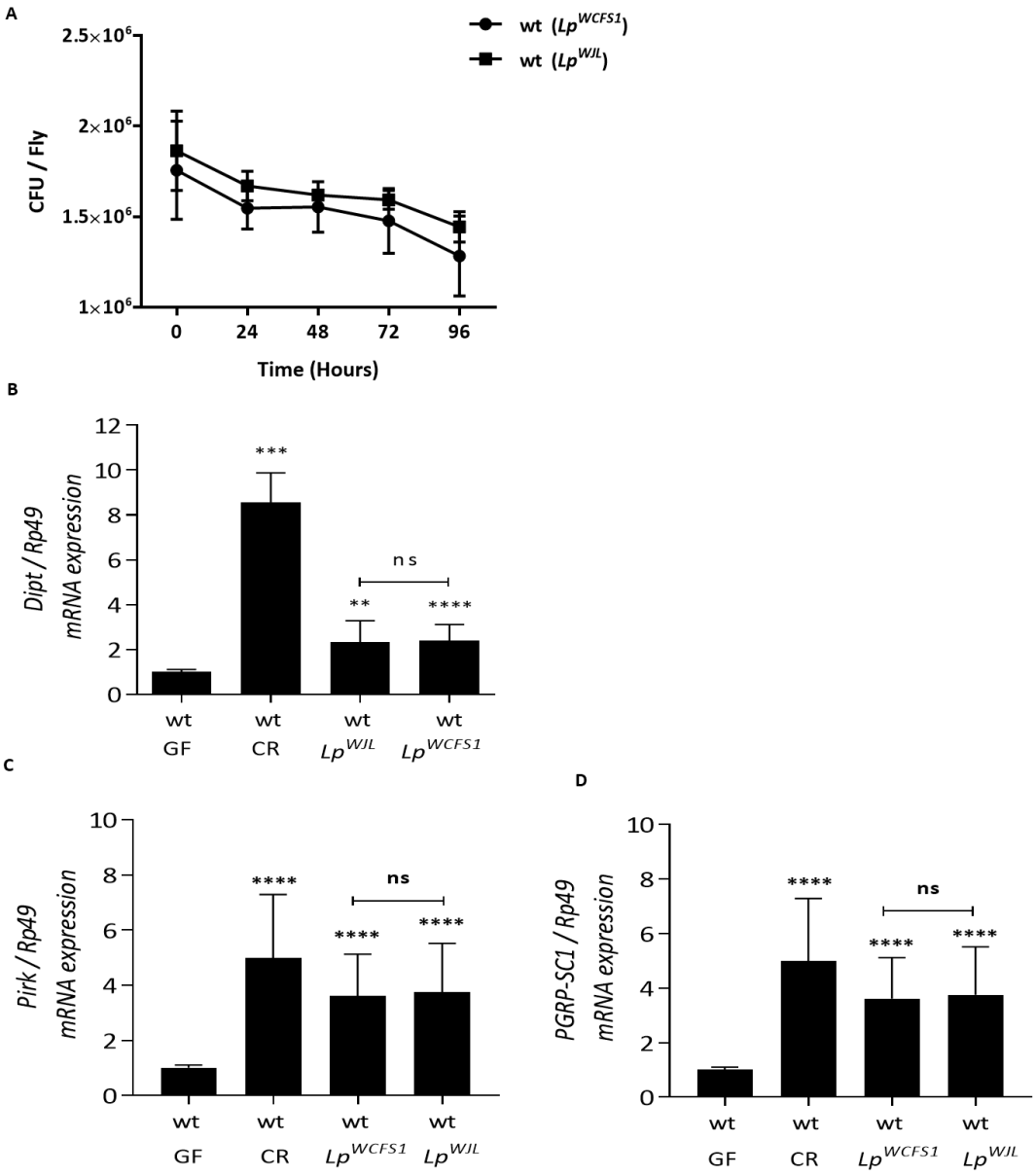

Figure 3 – figure supplement 1: *Lp*^{WCFS1} strain shares with *Lp*^{WJL} strain the ability to colonize the *Drosophila* gut and to induce the IMD pathway.

(A) Internal bacterial loads retrieved from the guts of Germ Free (GF) wild-type (wt) flies fed with *Lp*^{WCFS1} or *Lp*^{WJL} for three days. Colony Forming Unit (CFU) counting was performed every 24 hours after transferring the monoassociated flies to a fresh cornmeal medium vial.

(B, C and D) Relative expression of the *Diptericin* (*Dipt*) (B) *Pirk* (C) and *PGRP-SC1* (D) transcripts in the midgut of Germ Free (GF) wild-type (wt) flies fed with *Lp*^{WCFS1} or *Lp*^{WJL} for 3 days. Transcripts expression was measured by RT-qPCR in total RNA extracts. Ribosomal protein 49 (Rp49) transcript was used as reference gene. Transcripts levels detected in GF flies is set as a control.

Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $\underline{p} > 0.05$; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

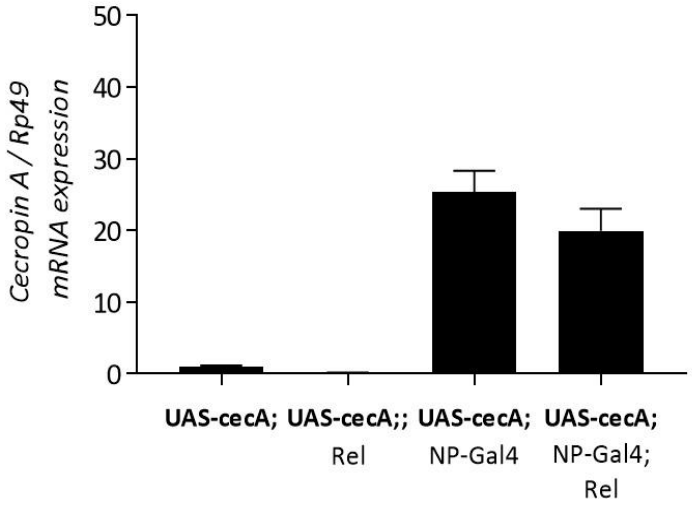

Figure 3 – figure supplement 2: Overexpression of *Cecropin* A gene in the gut of adult flies.

The expression of a *cecropin A* transgene (UAS-cec) was driven using a midgut specific driver, NP-Gal4, in wild-type (wt) or *Rel* mutant flies. The *cecropin A* expression a was quantified by RT-qPCR from the RNA extracts of dissected midguts of adult flies following their incubation for 3 days at 29 °C. Wt flies carrying the UAS-cec transgene were used as control. Data obtained from three independent experiments are combined in single value (mean ± sd).

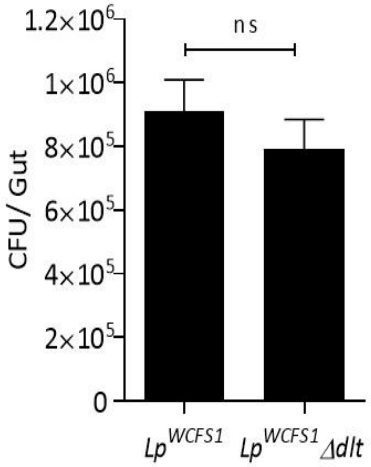

Figure 3 – figure supplement 3: Internal bacterial loads retrieved from the guts of Germ Free (GF) wild-type flies fed with *Lp*^{WCFS1} or *Lp* $^{WCFS1\Delta dlt}$ for three days.

Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$)

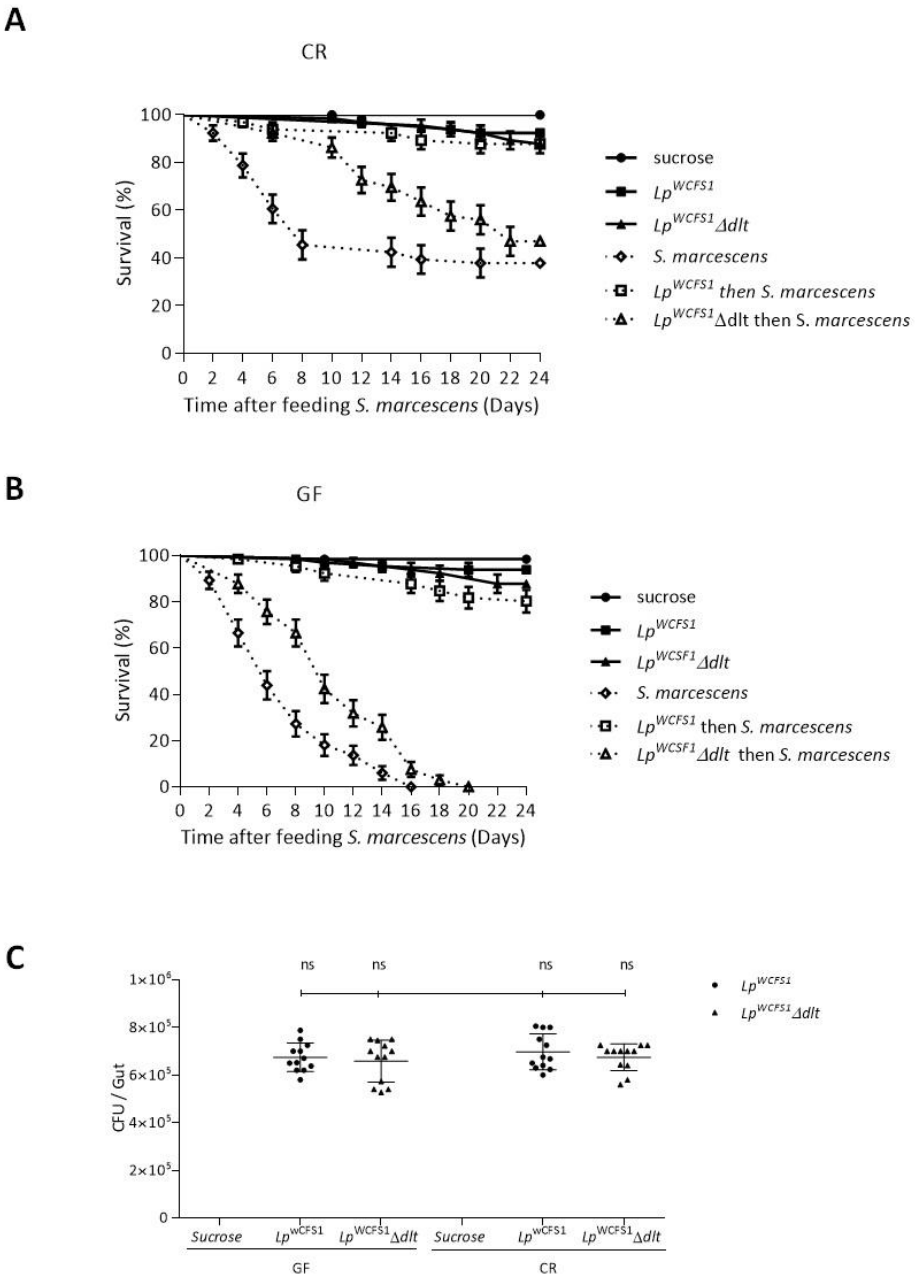

Figure 3 – figure supplement 4: D-alanylation of WTAs are essential for the probiotic effect of *Lp* ^{WCFS1} in *Drosophila*.

(A and B) Conventionally reared (CR) (A) and Germ Free (GF) (B) wt flies were fed with *Lp*^{WCSF1} or *Lp* ^{WCFS1}*Δdlt* for 3 days prior to *S. marcescens* challenge. Flies were fed a sucrose suspension of *S. marcescens* for 2 days and then transferred to a fresh cornmeal vial.

(C) Internal bacterial loads retrieved from the guts of Germ Free (GF) or conventionally reared (CR) wt flies fed with *Lp*^{WCFS1} or *Lp* ^{WCFS1}*Δdlt* for three days.

Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$).

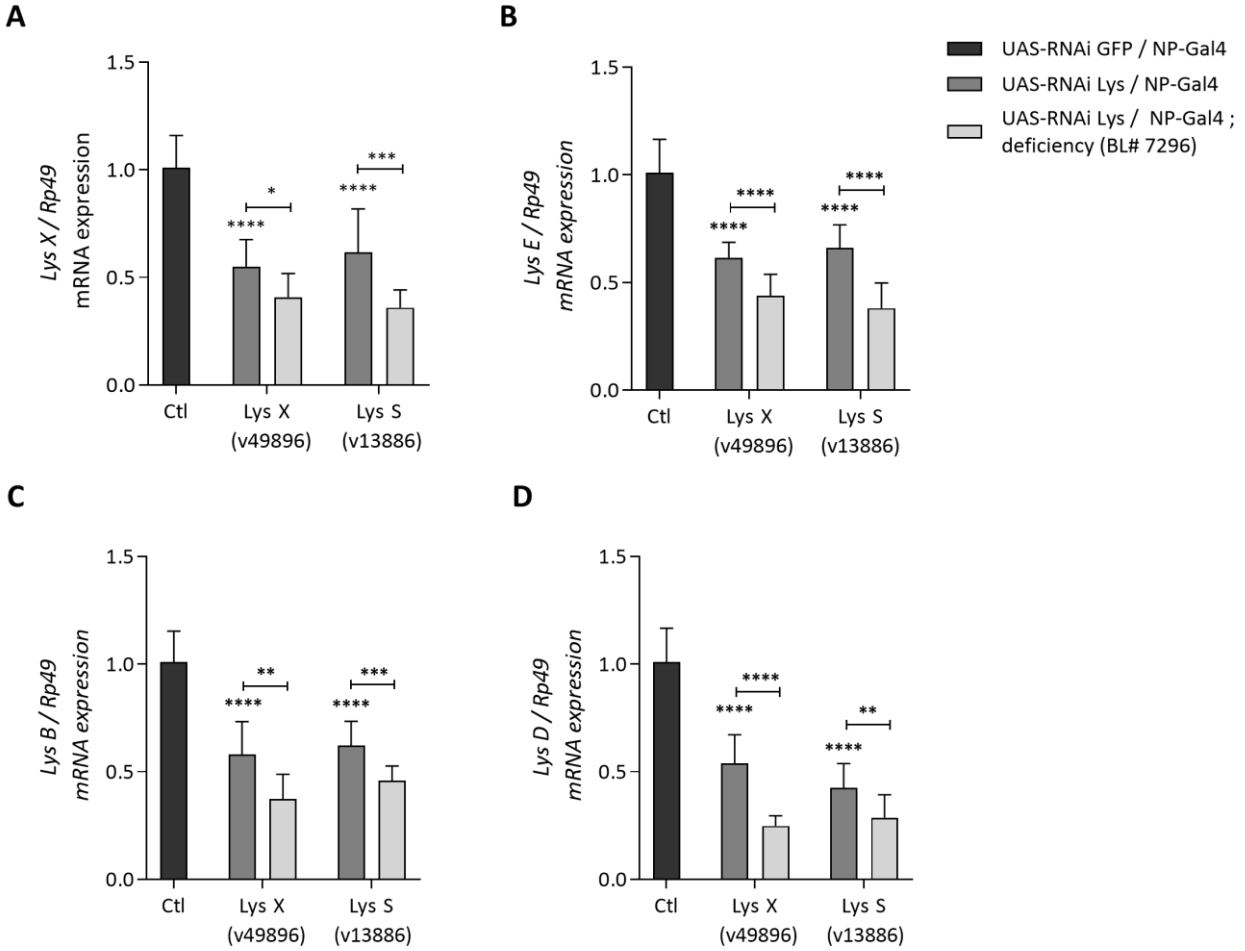

Figure 5 – figure supplement 1: RNAi silencing of *lysozyme* genes in the gut.

Silencing of the lysozyme genes was performed by the expression of RNAi constructs (UAS-RNAi) in the gut of adult flies using the midgut specific driver NP-Gal4. Two different transgenic fly lines (v49896) and (v13886) were used. These harbor different RNAi construct targeting the *LysX* or the *LysS* transcripts respectively. Silencing of lysozyme genes is further aggravated by crossing these transgenic flies to a fly line carrying a chromosomal deletion (deficiency BL#7296) uncovering the Lysozyme locus in the *Drosophila* genome. A transgenic line expressing an RNAi construct targeting the GFP transcript is used as a control. Relative expression of the *Lysozymes X* (A), *E* (B), *B*(C) and *D* (D) transcripts is measured in total RNA extracts from the midgut of the transgenic flies of the different genetic background Ribosomal protein 49 (Rp49) transcript was used as reference gene. Transcripts levels are compared to that detected in UAS-RNAi GFP transgenic flies that is arbitrary set to 1.
Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).