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

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

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15 # Abstract

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

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27 ## Introduction

28 The external surface of microorganisms is a central platform for interactions with multicellular
29 hosts. Evolutionarily selected Pattern Recognition Receptors (PRRs) of the innate immune
30 system sense key microbial surface molecules such as fungal glucans, Gram-negative bacterial
31 lipopolysaccharide (LPS), Gram-positive bacterial Peptidoglycan (PGN) and Teichoic Acids
32 (TAs) to alert the host of the microbial non-self and to initiate immune responses (Medzhitov,
33 2007). PRRs, such as the Toll-like receptors and NOD-like receptors act at the forefront of the
34 mucosal immune barrier. They control the expression of several immune induced genes such as
35 antimicrobial peptides (AMPs) and cytokines, the differential profiles of which dictate the
36 outcome of the ensued immune response (Hold, Mukhopadhya, & Monie, 2011). Therefore,
37 several hypotheses consider microbial surface molecules as plausible candidates for effector
38 probiotic molecules (Kleerebezem et al., 2010). Likewise, several pathogens have evolved
39 specialized mechanisms to modify their external surface in order to avoid detection or to develop
40 resistance to potent, highly conserved, immune effectors such as AMPs and lysozymes (Anaya-
41 Lopez, Lopez-Meza, & Ochoa-Zarzosa, 2013; Bechinger & Gorr, 2017; Koprivnjak & Peschel,
42 2011; Ragland & Criss, 2017). In humans, both lysozyme and AMPs are expressed at surface
43 epithelial cells as well as in phagocytes and thus act at the frontline of the host defenses
44 (Dommett, Zilbauer, George, & Bajaj-Elliott, 2005). Most AMPs are small cationic molecules
45 with a pronounced amphipathy, a property that accounts, as commonly agreed, for their
46 membrane targeting microbicidal activity. According to the model, AMPs are preferentially
47 attracted to negatively charged surfaces of the microbial cell envelopes where they get embedded
48 into the hydrophobic regions of the lipid membranes, thereby leading to membrane
49 destabilization and ultimately to cell death (Joo, Fu, & Otto, 2016). Lysozyme comprises an
50 evolutionarily conserved family of bactericidal proteins that are found in species ranging from
51 bacteriophages to man (Dommett et al., 2005). Their canonical killing mechanism relies on their

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52 muramidase activity, which cleaves the β-(1,4)-glycosidic bond between N-acetylmuramic acid
53 and N-acetylglucosamine moieties of the bacterial cell wall peptidoglycan. Resulting loss of cell
54 wall integrity leads to rapid cell lysis and death (Callewaert & Michiels, 2010; Ragland & Criss,
55 2017). The cationic nature of lysozyme is very important for bactericidal activity since it favors
56 its binding to the negatively charged bacterial cell envelop and hence access to the PGN
57 substrate.

58 In addition to the thick layer of PGN, the cell wall of Gram-positive bacteria is characterized by
59 the presence of highly charged anionic polymers of repeating alditol phosphate residues called
60 Teichoic acids (TAs). TAs represent the most abundant component of Gram-positive bacterial
61 cell wall and play crucial roles in their pathogenesis (Brown, Santa Maria, & Walker, 2013; Joo
62 et al., 2016). Notably, modification of TAs by addition of positively charged D-alanine esters
63 residues reduces their net negative charge thus lowering the attraction of cationic antimicrobial
64 peptides to the bacterial cell wall. This process is performed by the gene products of the *dlt*
65 operon that is highly conserved in most Gram-positive bacteria including probiotic and
66 pathogenic species (Abachin et al., 2002; Abi Khattar et al., 2009; Heaton & Neuhaus, 1992;
67 Kristian et al., 2005; Perego et al., 1995; Peschel et al., 1999). According to currently available
68 genome sequences, the *dlt* operon comprises four to five genes, *dltXABCD* (Abi Khattar et al.,
69 2009; Kamar et al., 2017; Perego et al., 1995). DltA is a D-alanine-D-alanyl carrier protein ligase
70 that catalyzes the D-alanylation of the D-alanyl carrier protein DltC (Debabov et al., 1996;
71 Heaton & Neuhaus, 1992, 1994). The roles of DltX, DltB and DltD are less clear (Debabov,
72 Kiriukhin, & Neuhaus, 2000; Kamar et al., 2017; Neuhaus & Baddiley, 2003). Nevertheless, in
73 all tested Gram-positive bacteria species, genetic disruption of any of the five genes of the *dlt*
74 operon completely abrogates its function. Compared to wild-type strains, *dlt* mutants have a
75 higher negative charge on the cell surface and are significantly susceptible to cationic
76 antimicrobial effectors including AMPs and lysozyme (Fabretti et al., 2006; Kovacs et al., 2006;

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

83 *Drosophila* has long emerged as a well-suited model for the study of host-pathogen interactions
84 (Ferrandon, 2013; Lemaitre & Hoffmann, 2007). The hallmark of the *Drosophila* host defense is
85 the immune-induced expression of genes encoding potent AMPs (Ferrandon, Imler, Hetru, &
86 Hoffmann, 2007; Lemaitre & Hoffmann, 2007). These peptides are synthesized by the fat body
87 cells (the fly immune organ) and secreted into the hemolymph where they constitute the main
88 effectors of the humoral systemic response. AMPs are also produced locally at the epithelial
89 barriers such as the gut, where they act synergistically with ROS to limit the infections
90 (Ferrandon, 2013; Imler & Bulet, 2005). Two highly conserved NF-κB signaling cascades, the
91 Toll and the Immune Deficiency (IMD) pathways, control the expression of AMP genes.
92 Whereas both pathways contribute to the regulation of the systemic immune response, only the
93 latter regulates the expression of AMP genes in the gut through the activation of the NF-κB
94 transcription factor Relish (El Chamy, Matt, Ntwasa, & Reichhart, 2015; Ferrandon, 2013). The
95 IMD pathway is elicited upon the sensing of DAP-type PGN fragments by members of the
96 Peptidoglycan Recognition Protein (PGRP) receptors family; the membrane bound PGRP-LC
97 and the intracellular PGRP-LE (Chang, Chelliah, Borek, Mengin-Lecreulx, & Deisenhofer,
98 2006; Chang et al., 2005; Choe, Lee, & Anderson, 2005; Choe, Werner, Stoven, Hultmark, &
99 Anderson, 2002; Gottar et al., 2002; Kaneko et al., 2004; Kaneko et al., 2006; Leulier et al.,
100 2003; Mellroth & Steiner, 2006; Ramet, Manfruelli, Pearson, Mathey-Prevot, & Ezekowitz,
101 2002). Potential immune effectors, such as lysozymes are also expressed at the epithelial barrier

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102 (Daffre, Kylsten, Samakovlis, & Hultmark, 1994). However, their global contribution to the host
103 defense and intestinal homeostasis remains obscure. More recently, *Drosophila* has become a
104 powerful model for the characterization of the molecular mechanisms underlying the mutualistic
105 host-microbiota interactions (Erkosar & Leulier, 2014; W. J. Lee & Brey, 2013; Ma, Storelli,
106 Mitchell, & Leulier, 2015). Indeed, several studies have proven the relative simplicity of the fly
107 intestinal microbiota, which comprises approximately 30 phylotypes with a major representation
108 of *Lactobacillaceae* and *Acetobacteraceae* (Blum, Fischer, Miles, & Handelsman, 2013;
109 Chandler, Lang, Bhatnagar, Eisen, & Kopp, 2011; Corby-Harris et al., 2007; Cox & Gilmore,
110 2007; Staubach, Baines, Kunzel, Bik, & Petrov, 2013; C. N. Wong, Ng, & Douglas, 2011).
111 Reports have begun to illustrate the impact of this microbial community on the *Drosophila* host
112 biology (Buchon, Broderick, Chakrabarti, & Lemaitre, 2009; Iatsenko, Boquete, & Lemaitre,
113 2018; Jones et al., 2013; Sharon et al., 2010; Shin et al., 2011; Storelli et al., 2011; A. C. Wong,
114 Dobson, & Douglas, 2014). Notably, the gut commensals largely influence the *Drosophila*
115 midgut transcriptome and promote the expression of genes associated with gut physiology and
116 metabolism as well as tissue homeostasis and immune defenses (Broderick, Buchon, & Lemaitre,
117 2014; Erkosar & Leulier, 2014). Interestingly, some *Lactobacillus plantarum* strains recapitulate
118 the benefits of a *bona fide Drosophila* commensal thus providing a simple model for the
119 investigation of the intricate mechanisms underlying the impact of intestinal bacteria on host
120 physiology and mucosal immunity (Jones et al., 2013; Storelli et al., 2011). In this context, high
121 throughput analyses have shown that the gut-associated bacteria set an immune barrier in the
122 *Drosophila* gut through the activation of a transcriptional program that is largely dependent on
123 the IMD pathway (Bosco-Drayon et al., 2012; Broderick et al., 2014; Buchon et al., 2009; Ryu et
124 al., 2008). Remarkably though, the expression of AMP genes remains at its basal level compared
125 to that induced upon an oral infection (Bosco-Drayon et al., 2012) and any alteration in this
126 transcriptional profile leads to commensal dysbiosis, which dramatically affects the host fitness

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127 and lifespan (Bonnay et al., 2013; Broderick et al., 2014; Ryu et al., 2008). Recent studies have
128 shown that the immune response is compartmentalized in the *Drosophil*a gut (Bosco-Drayon et
129 al., 2012; Buchon, Broderick, & Lemaitre, 2013) and that multiple negative regulators intervene
130 at different levels of the IMD pathway in order to fine-tune its activation (Aggarwal &
131 Silverman, 2008; Bischoff et al., 2006; Guo, Li, & Lin, 2014; Kleino et al., 2008; Lhocine et al.,
132 2008; Mellroth, Karlsson, & Steiner, 2003; Paredes, Welchman, Poidevin, & Lemaitre, 2011;
133 Ryu et al., 2004; Zaidman-Remy et al., 2006). Among these negative regulators are catalytic
134 members of the PGRP family, such as PGRP-SC and PGRP-LB, which degrade PGN fragments
135 into non-immunostimulatory moieties and the intracellular protein, Pirk, which binds to the
136 PGRP-LC and PGRP-LE receptors thus disrupting downstream signaling complex (Aggarwal et
137 al., 2008; Kleino et al., 2008; Kleino et al., 2017; Lhocine et al., 2008). However, similarly to
138 infectious bacteria, *L. plantarum* and the major elements of the fly microbiota produce DAP-type
139 PGN and most of the IMD negative regulators act in a feedback loop. Thus, what makes this
140 microbial community only a mild inducer of the AMP genes and how it is tolerated in the
141 *Drosophila* gut remains intriguing (Bosco-Drayon et al., 2012).

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

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151 # Results

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

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

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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).

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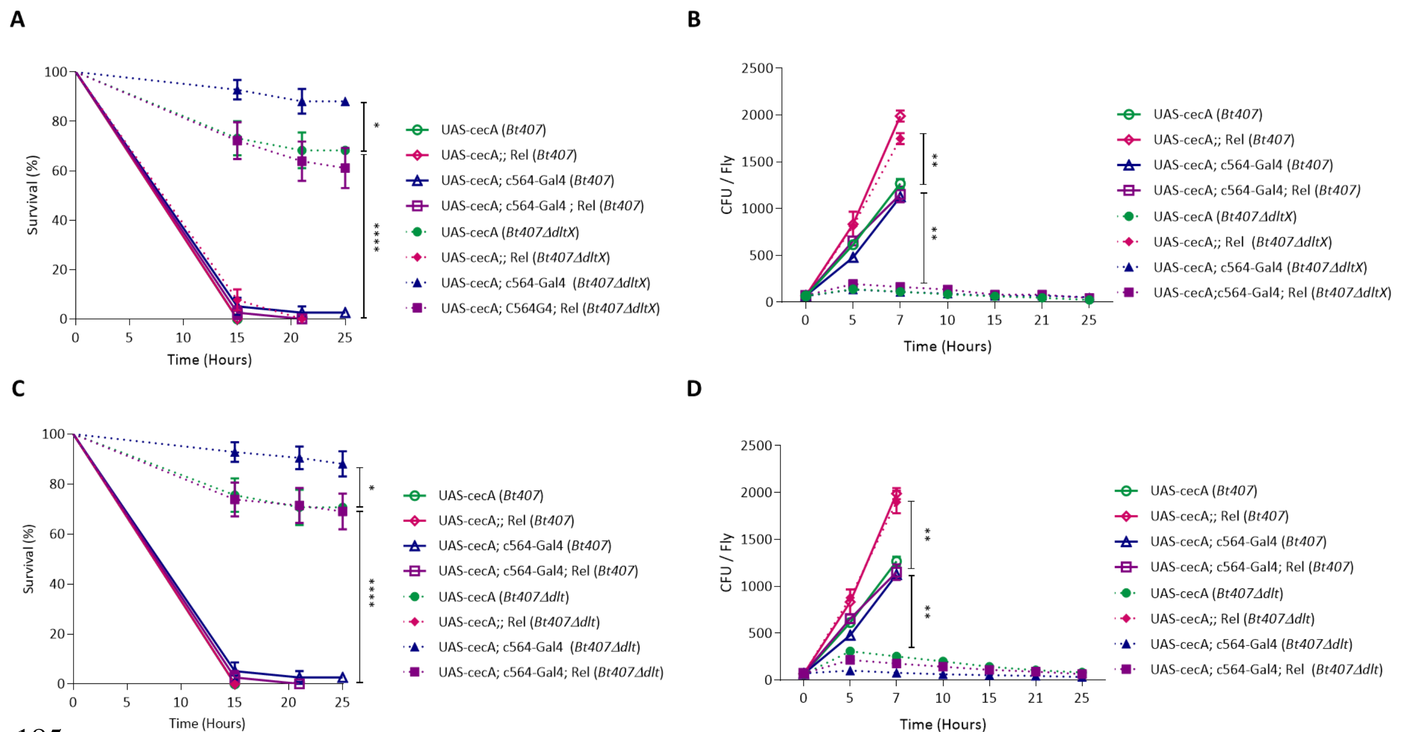


185

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

188 (A and C) Survival of adult wild-type (wt) or relish (*Rel*) mutant, overexpressing or not a *Cecropin A* transgene
189 (under the control of the c564-Gal4 fat body driver) to an infection with *Bt407, Bt407ΔdltX* (A) and *Bt407Δdlt* (C).
190 (B and D) Internal bacterial loads retrieved from adult flies infected with *BT407*and *Bt407ΔdltX* (B) or Bt407*Δdlt*
191 (D). The Colony Forming Unit (CFU) counting was performed only on survival flies.
192 Data are pooled from three independent experiments (mean and s.d.). Statistical tests were performed using the Log
193 Rank test for the survival assays and Mann-Whitney test for the CFU counting within Prism software (ns: $p > 0.05$;
194 * $0.01 < p < 0.05$; **: $0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).The exact *p* values are listed in
195 Figure 1 – source data 1.

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

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

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

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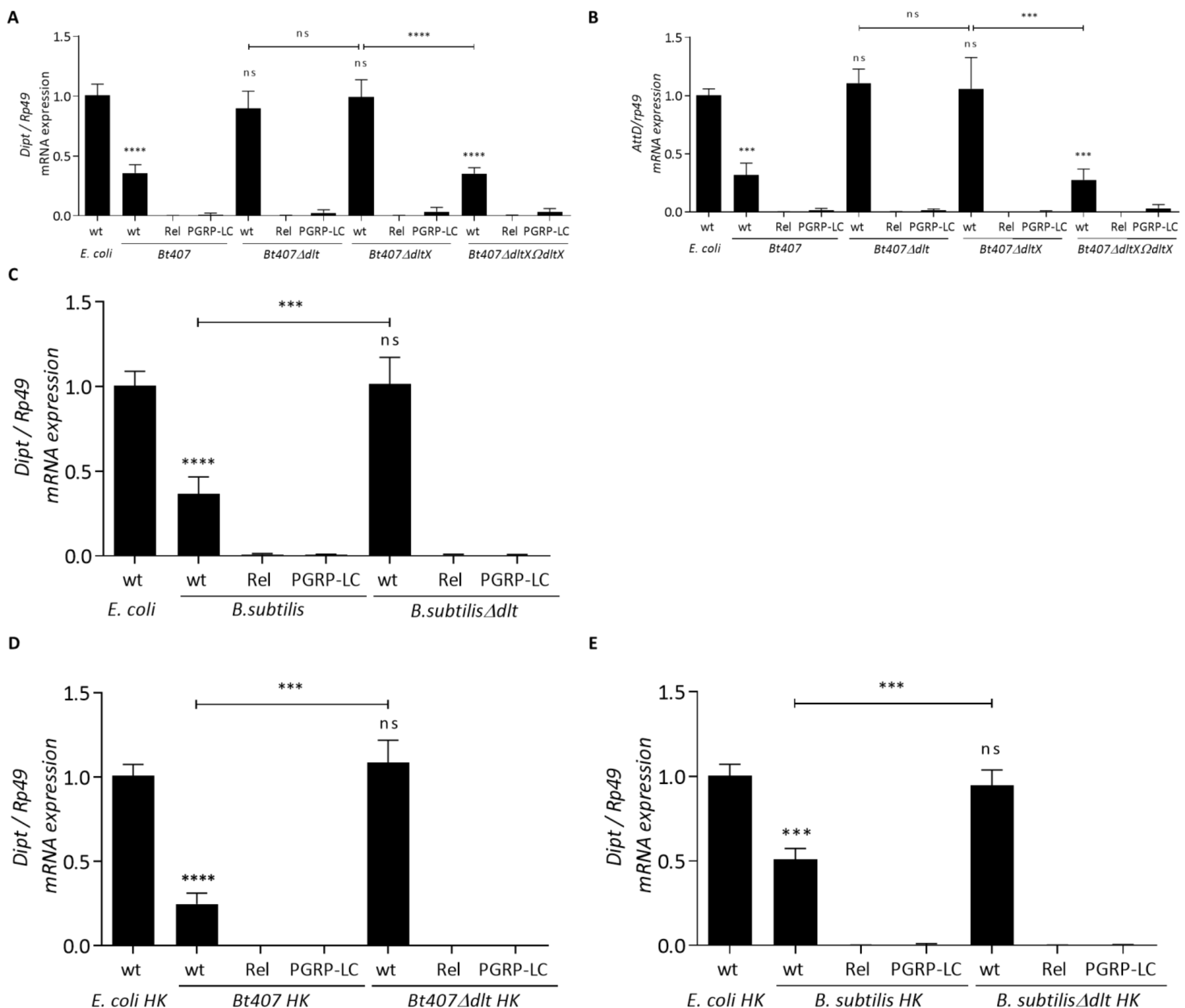


229

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

232 Relative expression of the *Diptericin* (*Dipt*) (A, C D and E) or *Attacin D* (*AttD*) (B) transcripts in wild-type (wt) or
233 *relish* (*Rel*) mutant flies induced by living or heat-killed *Bt407, Bt407Δdlt, Bt407ΔdltX* and *Bt407ΔdltXΩdltX (A, B*
234 *and D) or B. subtilis (C and E).* Transcripts expression was measured by RT-qPCR in total RNA extracts 4 hours
235 upon the induction. Ribosomal protein 49 (Rp49) transcript was used as reference gene. Transcripts levels are
236 compared to that triggered in wt flies infected by *E. coli* as a control.
237 Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were
238 performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$;
239 ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).The exact $p$ values are listed in Figure 2 – source data 1.

240 The following source data is available for figure 2:
241 **Source data 1.** $p$ values for figure 2

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

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

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

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292 3A, the counts of the wt *Lp*$^{WCFS1}$ strain were constant in the guts of the recolonized flies despite
293 the overexpression of Cecropin, whereas those of the *Lp*$^{WCFS1\Delta dlt}$ mutant strain dramatically
294 declined in less than four days. Altogether, these data underline a role of the *dlt* operon in the
295 resistance of *Lp*$^{WCSF1}$ to cationic antimicrobial effectors thus favoring its persistence in the
296 *Drosophila* gut.

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

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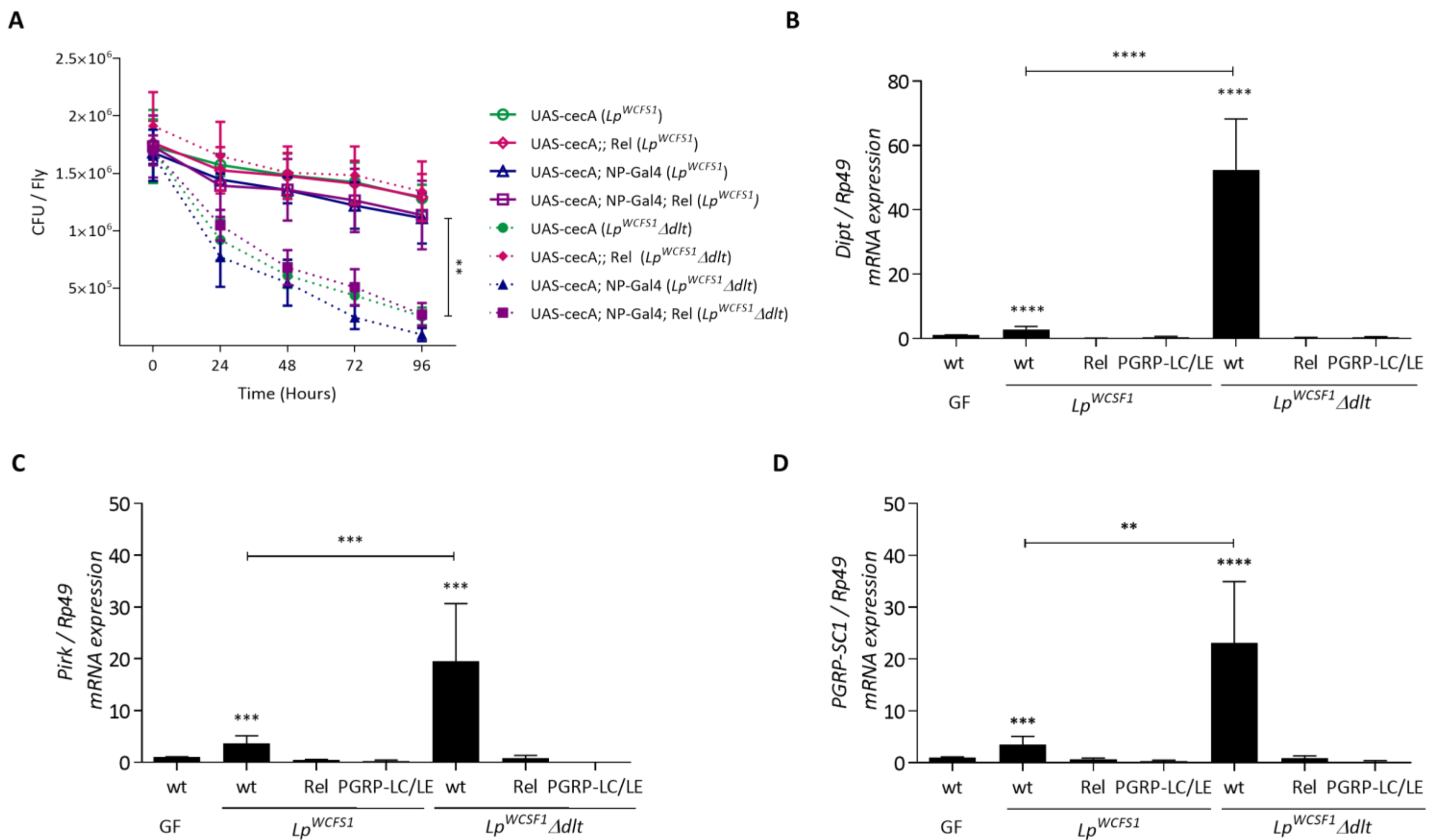


313

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

316 (A) Internal bacterial loads retrieved from the guts of Germ Free (GF) wild-type (wt) or *relish* (Rel) mutant flies fed
317 with $Lp^{WCFS1}$ or $Lp^{WCFS1}\Delta dlt$ for three days. The expression of a *Cecropin* transgene is specifically driven in the gut
318 of these flies by the NP-Gal4 driver. Colony Forming Unit (CFU) counting was performed every 24 hours after
319 transferring the monoassociated flies to a fresh cornmeal medium vial.
320 (B, C and D) Relative expression of the *Diptericin* (*Dipt*) (B) *Pirk*) (C) and *PGRP-SC1* (D) transcripts in the midgut
321 of Germ Free (GF) wild-type (wt), *relish* (*Rel*) or *PGRP-LC/LE* double mutants flies fed with $Lp^{WCFS1}$ or $Lp^{WCFS1}\Delta dlt$
322 for 3 days. Transcripts expression was measured by RT-qPCR in total RNA extracts. Ribosomal protein 49 (Rp49)
323 transcript was used as reference gene. Transcripts levels detected in GF flies is set as a control.
324 Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were
325 performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$;
326 ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$). The exact *p* values are listed in Figure 3 – source data 1.

327 The following source data and figure supplements are available for figure 3:
328 **Source data 1.** P values for figure 3
329 **Figure supplement 1**. $Lp^{WCFS1}$ strain shares with $Lp^{WJL}$ strain the ability to colonize the *Drosophila* gut and to
330 induce the IMD pathway.
331 **Figure supplement 2.** Overexpression of *Cecropin* A gene in the gut of adult flies.
332 **Figure supplement 3.** Internal bacterial loads retrieved from the guts of Germ Free (GF) wild-type flies fed with
333 $Lp^{WCFS1}$ or $Lp^{WCFS1\Delta dlt}$ for three days.
334 **Figure supplement 4.** D-alanylation of WTAs are essential for the probiotic effect of $Lp^{WCFS1}$ in *Drosophila*.

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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

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

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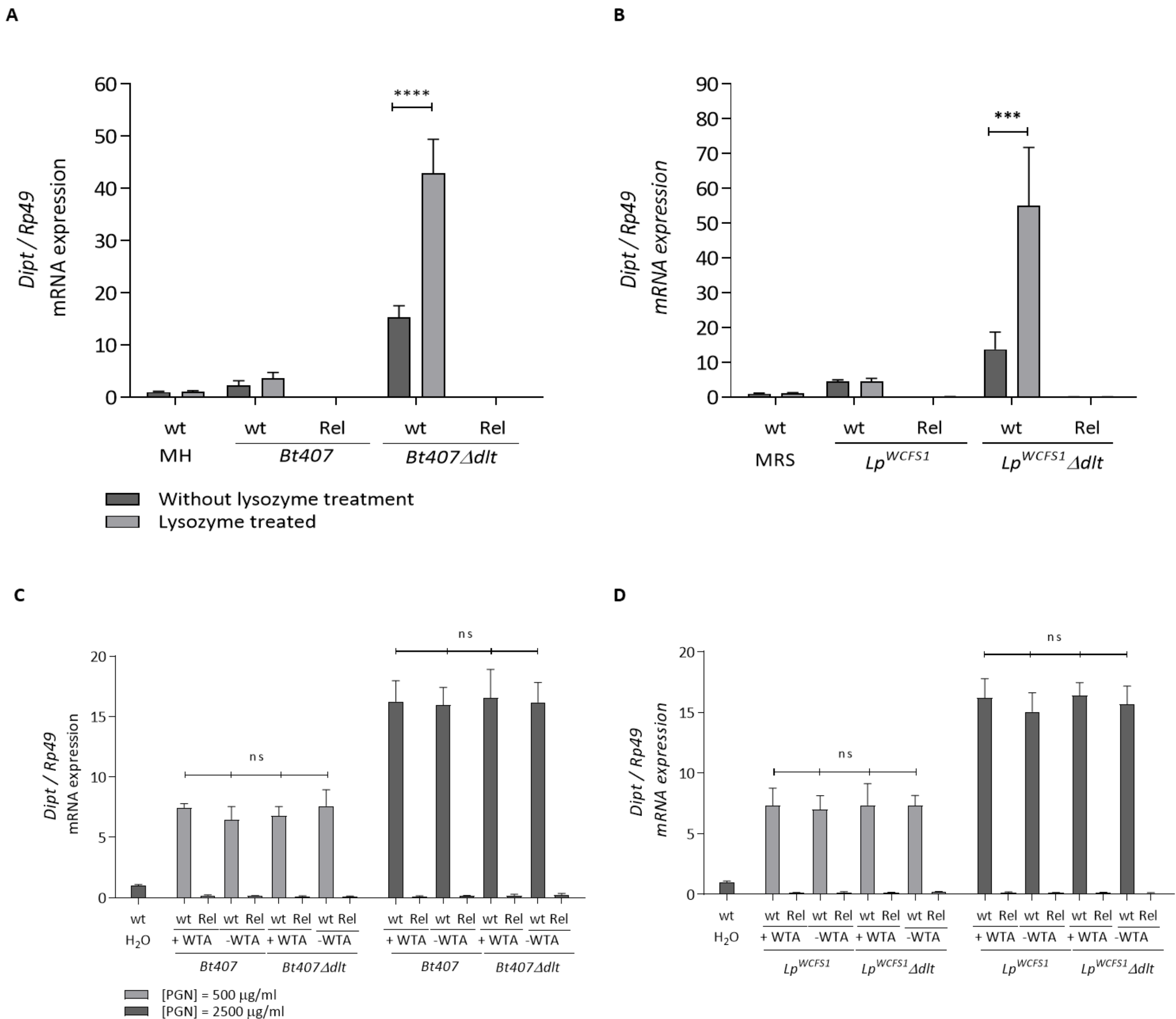


372

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

375 (A and B) Wild-type (wt) and *relish* (Rel) mutant flies were injected with the supernatant of *Bt407* and $Lp^{WCSF1}$
376 cultures or their corresponding *dlt* mutants (*Bt407Δdlt* and $Lp^{WCSF1}$*Δdlt)* treated or not by chicken egg lysozyme (10
377 mg/ml).
378 (C and D) Wild-type (wt) and *relish* (Rel) mutant flies were injected with cell wall peptidoglycan (PGN) fractions
379 with or without cell wall TAs (500 and 2500 µg/ml) from the wild type or the *dlt* mutants of*Bt407* and $Lp^{WCSF1}$.
380 Relative *Dipptericin* (dipt) expression was measured by RT-qPCR 4 h after the microbial induction of the adult flies.
381 Ribosomal protein 49 (Rp49) transcript was used as reference gene. Transcripts levels detected in wt flies injected
382 with MH Broth medium (A), MRS Broth Medium (B) or water (C and D) are set as controls.
383 Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were
384 performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; *: $0.01 < p < 0.05$; **: $0.001 < p <$
385 $0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$). The exact p values are listed in Figure 4 – source data 1.

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

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

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

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

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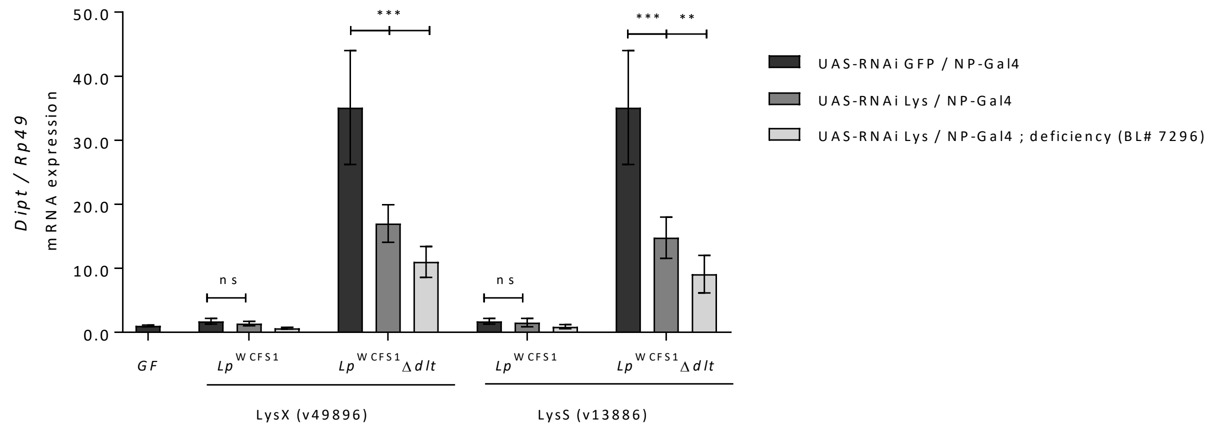


421

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

424 Silencing of the lysozyme genes was performed by the expression of RNAi constructs (UAS-RNAi) in the gut of
425 adult flies using the midgut specific driver NP-Gal4. Two different transgenic fly lines (v49896) and (v13886) were
426 used. These harbor different RNAi construct targeting the *LysX* or the *LysS* transcripts respectively. Silencing of
427 *lysozyme* genes is further aggravated by crossing these transgenic flies to a fly line carrying a chromosomal deletion
428 (deficiency BL#7296) uncovering the *lysozyme* locus in the *Drosophila* genome. A transgenic line expressing an
429 RNAi construct targeting the GFP transcript is used as a control. Relative expression of the *Diptericin* transcript is
430 measured in total RNA extracts from the midgut of Germ Free wild-type flies (GF) or the transgenic flies of the
431 different genetic background fed on $Lp^{WCFS1}$ or $Lp^{WCFS1}\Delta dlt$ for 3 days. Ribosomal protein 49 (Rp49) transcript was
432 used as reference gene. Transcripts levels are compared to that detected in in GF flies that is arbitrary set to 1.
433 Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were
434 performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; *: $0.01< p < 0.05$; **: $0.001< p < 0.01$;
435 ***: $0.0001< p < 0.001$; ****: $p < 0.0001$). The exact p values are listed in Figure 5 – source data 1.

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

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439 **Discussion**

440 The role of the *dlt*-operon in mediating resistance to cationic antimicrobial effectors *in vitro* has
441 been attested for several Gram-positive bacteria species (Fabretti et al., 2006; Kovacs et al.,
442 2006; Kristian et al., 2005; Perea Velez et al., 2007). This attribute is associated with an
443 attenuated virulence of the *dlt* mutants for several pathogens including *Listeria monocytogenes*,
444 *Bacillus anthracis* and *Staphylococcus aureus* (Abachin et al., 2002; Collins et al., 2002; Fisher
445 et al., 2006; Kristian et al., 2003; Tabuchi et al., 2010). Only for the latter, a role for the *dlt*-
446 operon in hampering immune sensing and the subsequent activation of the Toll pathway in
447 *Drosophila* has been proposed (Tabuchi et al., 2010). The Toll pathway is preferentially
448 triggered upon the sensing of Lys-type PGN by a receptor complex comprising PGRP-SA and
449 GNBP1, a member of the *Drosophila* Glucan-Binding protein family (Gobert et al., 2003;
450 Michel, Reichhart, Hoffmann, & Royet, 2001). However, although the exact mechanism
451 underlying the inhibitory effect of D-alanine esterification of TAs on the Toll pathway activation
452 remains unknown, the results obtained by Kurokawa *et al*. suggest that it entails the covalent
453 bonding of D-alanylated Wall TAs to PGN (Kurokawa et al., 2011). This model is challenged by
454 a report from Atilano *et al.* which indicates that wall TAs limit the access of PGN to the PGRP-
455 SA receptor independently from the activity of the *dlt*-operon (Atilano, Yates, Glittenberg,
456 Filipe, & Ligoxygakis, 2011). We show here that, the presence of TAs, whether D-alanylated or
457 not, does not alter the IMD response upon injection of purified PGN preparations in flies (Figure
458 4B). Furthermore, the quantification of the IMD response in flies stimulated by injection of
459 supernatants of bacterial cultures treated or not by chicken egg lysozyme, or increasing
460 concentration of PGN preparations, reveals a correlation between the induced response and the
461 doses of injected PGN fragments (Figure 4 A and B). Overall, our data support a model in which
462 the differential immune response following infection of flies by wt or *dlt* mutant bacteria is
463 linked to variations in the quantities of released PGN that are made available for sensing by the

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

467 Resistance to antimicrobial effectors and evasion from immune detection are prominent features
468 of pathogenic bacteria. Interestingly, our data indicate that the D-alanylation of TAs is a common
469 microbial strategy employed by pathogen and commensal alike to elude and resist immune
470 defenses thus acquiring the capacity to develop or persist in appropriate niches. Indeed, our
471 results reveal the essential role of D-alanine esterification in the resistance of *L. plantarum* to the
472 assault of intestinal cationic lysozyme and thus for its persistence as a core component of the
473 *Drosophila* microbiota (Figure 3A). These findings are in agreement with a previous report
474 attesting of the prominence of D-alanine esterification of TAs for the colonization of *L. reuteri* in
475 the mouse intestinal tract (Perea Velez et al., 2007; Walter et al., 2007). The *dlt* operon was also
476 found to be an essential element for the establishment of *L. casei* in the gut of the rabbit ligated
477 ileal loop model (Licandro-Seraut, Scornec, Pedron, Cavin, & Sansonetti, 2014). Our data also
478 point the crucial role of the D-alanylation of TAs in maintaining the immunomodulatory
479 potential of *L. plantarum* in the *Drosophila* gut. This cell wall modification allows the fine-
480 tuning of IMD signaling as to set an essential immune barrier in the gut, which is a prerequisite
481 for the maintenance of a healthy commensal population. Indeed, several lines of evidence have
482 indicated the harmful effects of an exacerbated IMD response in the *Drosophila* gut (Bonnay et
483 al., 2013; Lhocine et al., 2008; Paredes et al., 2011; Ryu et al., 2008). Notably, a dysregulated
484 IMD response has been associated with commensal dysbiosis and epithelial dysplasia (Guo et al.,
485 2014; Ryu et al., 2008). These data further highlight the importance of the dual role of D-
486 alanylation of TAs in the resistance to antimicrobial effectors and the escape of immune
487 detection as defining characteristics of *L. plantarum* as a *bona fide* beneficial *Drosophila*
488 commensal. In other words, resistance to antimicrobial effectors allows the persistence of *L.*

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

495 Immunomodulation is as an essential property by which lactic acid bacteria exert their biological
496 functions in mammals. It is also the main criteria for the selection of probiotic strains (Bron,
497 Tomita, Mercenier, & Kleerebezem, 2013; I. C. Lee, Tomita, Kleerebezem, & Bron, 2013;
498 Wells, 2011). Notably, the cytokine profiles triggered upon the sensing of these bacteria in the
499 intestinal mucosa determine the subsequent polarization of T cells, and thus the orientation of the
500 ensuing adaptive immune response. Differential cytokine profiles are triggered upon the sensing
501 of appropriate Microbial Associated Molecular Patterns (MAMPs) of *lactobacilli* by cognate
502 PRRs at the intestinal epithelial barrier (Lebeer, Vanderleyden, & De Keersmaecker, 2010).
503 Notably, previous data have suggested that the sensing of lipoteichoic acid (LTA) is mainly
504 performed by the transmembrane TLR2 receptor, whereas sensing of bacterial PGN fragments is
505 preferentially mediated by intracellular NOD receptors (Macho Fernandez, Pot, & Grangette,
506 2011; Smelt et al., 2013; Travassos et al., 2004). Interestingly, the wt and the *dlt*-mutant of the
507 $Lp^{WCFS1}$ strain triggered a differential cytokine profiles in mammals (Grangette et al., 2005;
508 Smelt et al., 2013). The transcriptional response triggered by the former is marked by the
509 expression of TLR2-dependent pro-inflammatory IL-12 whereas that activated by the latter is
510 characterized by an enhanced expression of anti-inflammatory IL-10 that is mainly NOD-
511 dependent (Grangette et al., 2005; Macho Fernandez, Pot, et al., 2011; Macho Fernandez,
512 Valenti, et al., 2011). Similar results were obtained with a wt and *dlt*-mutant strain of *L.*
513 *rhamnosus GG* (Claes et al., 2010). Accordingly, the *dlt* mutants alleviated the inflammatory

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

526 Several reports have demonstrated the potential of *L. plantarum* to impact *Drosophila*
527 development and physiology (Blum et al., 2013; Bosco-Drayon et al., 2012; Broderick et al.,
528 2014; Erkosar & Leulier, 2014; Guo et al., 2014; Jones et al., 2013; Matos et al., 2017; Ryu et
529 al., 2008; Sharon et al., 2010; Storelli et al., 2011; A. C. Wong et al., 2014). Notably, work from
530 Leulier's laboratory revealed the importance of this bacterium in promoting juvenile growth
531 during chronic undernutrition (Storelli et al., 2011). Interestingly, Matos *et al.* showed that a
532 functional *dlt*-operon is required for this biological function of *L. plantarum* (Matos et al., 2017).
533 Our data give further evidence of the importance of the *dlt*-dependant D-alanylation of TAs for
534 the beneficial impacts of *L. plantarum* on its host biology in particular by tuning the sensing of
535 PGN immunostimulants, thus establishing an *ad hoc* balanced immune response at the intestinal
536 epithelium. Moreover, in *Drosophila* as in mammals, *L. plantarum* was shown to exert protective
537 probiotic effects. Notably, in a recent study, Blum *et al.* provided evidence of a protective role of
538 *L. plantarum* against intestinal pathogens such as *Serratia marcescens*. In this context,

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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.

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562 # Materials and Methods

563 **Bacterial strains and growth conditions**

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

571 The *dltA* mutant of *B. subtilis* was constructed in strain 168 as follows: an internal fragment of
572 the *dltA* gene was amplified with primers BSdltA-*Eco*RI (5'-ggaattcctcggccagcagattcagacagttt-
573 3') and BSdltA-BamH1 (5'-acccggatccatcaggcacatttg-3'). The PCR product and the integrative
574 plasmid pDIA5307 (PMID: 8113162) were digested with *Eco*RI and *Bam*HI and ligated together
575 generating pDA5307-BSdltA. The ligated product was digested with *Sma*I to linearize any
576 pDIA5307 plasmid left, transformed into competent cells of strain 168 and selected on
577 chloramphenicol (4 µg/ml). Clones were validated by PCR and 4 independent clones were stored
578 generating strain 168 Δ*dltA*. Clone 1 was used in this work.

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

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

587 Heat killing of bacteria was performed as described (El Chamy, Leclerc, Caldelari, & Reichhart,
588 2008) Briefly, bacterial solutions followed two steps of 20 minutes of incubation at 95°C
589 separated by 20 minutes of cooling on ice. Killing was verified by plating 100 µl of each
590 bacterial solution on LB agar plates.

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

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

597 Quantification of D-Ala esterified to TAs was performed by HPLC as described previously
598 (Kamar et al., 2017) , after release by alkaline hydrolysis of whole cells or cell wall fractions.
599 Cell walls were prepared from $Lp^{WCFS1}$, $Lp^{WCFS1}\Delta dlt$, *Bt407* and *Bt407Δdlt* as described (Bernard
600 et al., 2011). SDS, Pronase (2 mg/ml), Trypsin (200 µg/ml), DNase (50 µg/ml) and RNase (50
601 µg/ml) treatments were applied to obtain cell walls containing peptidoglycan and covalently
602 attached glycopolymers (WTA, polysaccharides). All incubations were performed at pH ≤ 6.0, to
603 keep D-Ala esterified on WTAs. The presence of D-Ala in the purified cell walls was checked by
604 HPLC as described above.
605 Purified peptidoglycan was obtained after incubation of cell walls in 48% hydrofluoric acid at
606 4°C for 16 hours, to remove WTA and other polysaccharides attached covalently to
607 peptidoglycan.

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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

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633 15, 21 and 25 hours post infection. The *Diptericin* expression was measured by RT-qPCR 4h
634 post infection.

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

639 9.2nl of sonicated purified peptidoglycan or 69 nl of supernatant treated with lysozyme were
640 injected with into the thorax of batches of 20 to 25 female flies (aged 2 to 4 days old) with a
641 Nanoject apparatus (Drummond, Broomall, PA). These flies were incubated 4 hours at 29 °C
642 then *Diptericin* expression was quantified in RNA extracts by RT-qPCR.

643 **Fly internal bacterial load quantification**

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

649 **Monoassociation of germ-free flies**

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

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

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

659 **Quantification of gene expression**

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

670 **Statistical analysis**

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

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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*.

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702 **Figure 5:**

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

## 704 Sources Data

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

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

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

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

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

## 710 Additional files

### 711 Supplementary files

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

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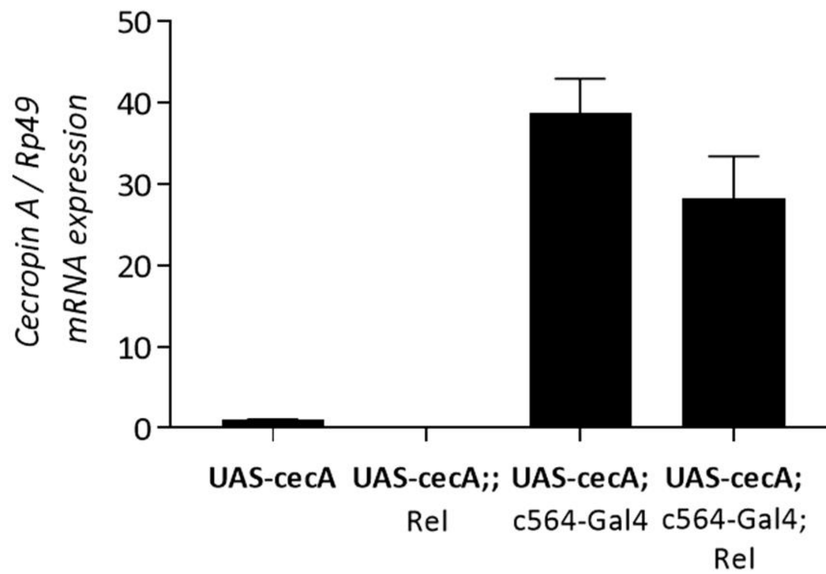
1 # Figures supplements




2

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

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

8

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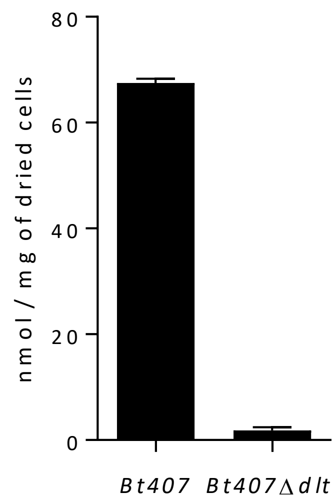


9

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

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

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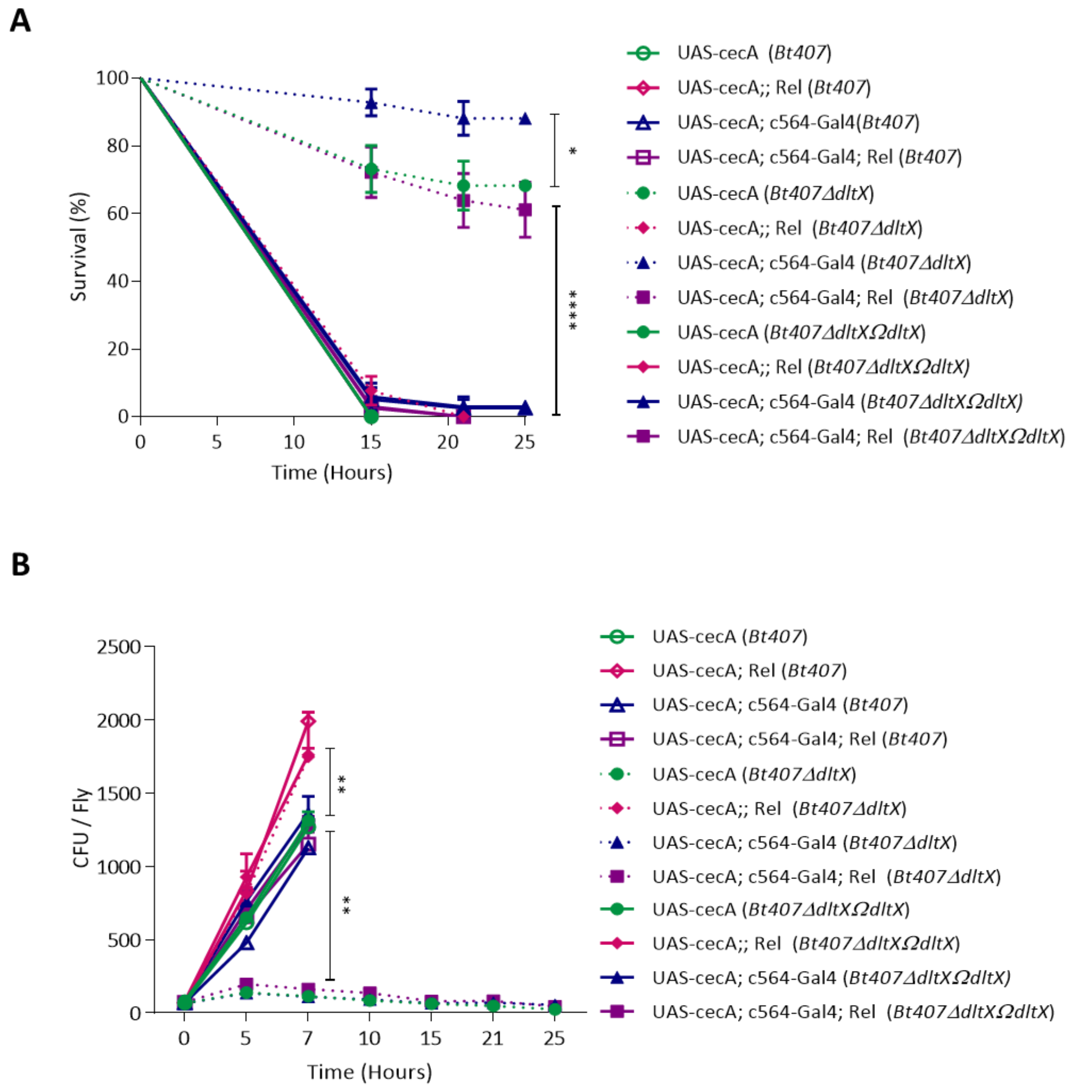


13

14 **Figure 1 – figure supplement 3: Complementation of the *Bt407ΔdltX* mutant with the *dltX-ORF*
15 (*Bt407ΔdltΩdltX*) completely restores its virulence similarly to the wt *Bt407* strain.**

16 (A) Survival of adult wild-type (wt) or relish (Rel) mutant, overexpressing or not a *Cecropin* transgene (under the
17 control of the c564-Gal4 fat body driver) to an infection with *Bt407, Bt407ΔdltX and Bt407ΔdltX* Ω*dltX*
18 (B) Internal bacterial loads retrieved from adult flies infected with *BT407*and *Bt407ΔdltX* or *Bt407ΔdltX* Ω*dltX*.
19 The Colony Forming Unit (CFU) counting was performed only on survival flies.
20 Data are pooled from three independent experiments (mean and s.d.). Statistical tests were performed using the
21 Log Rank test for the survival assays and Mann-Whitney test for the CFU counting within Prism software (ns: $p >$
22 $0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

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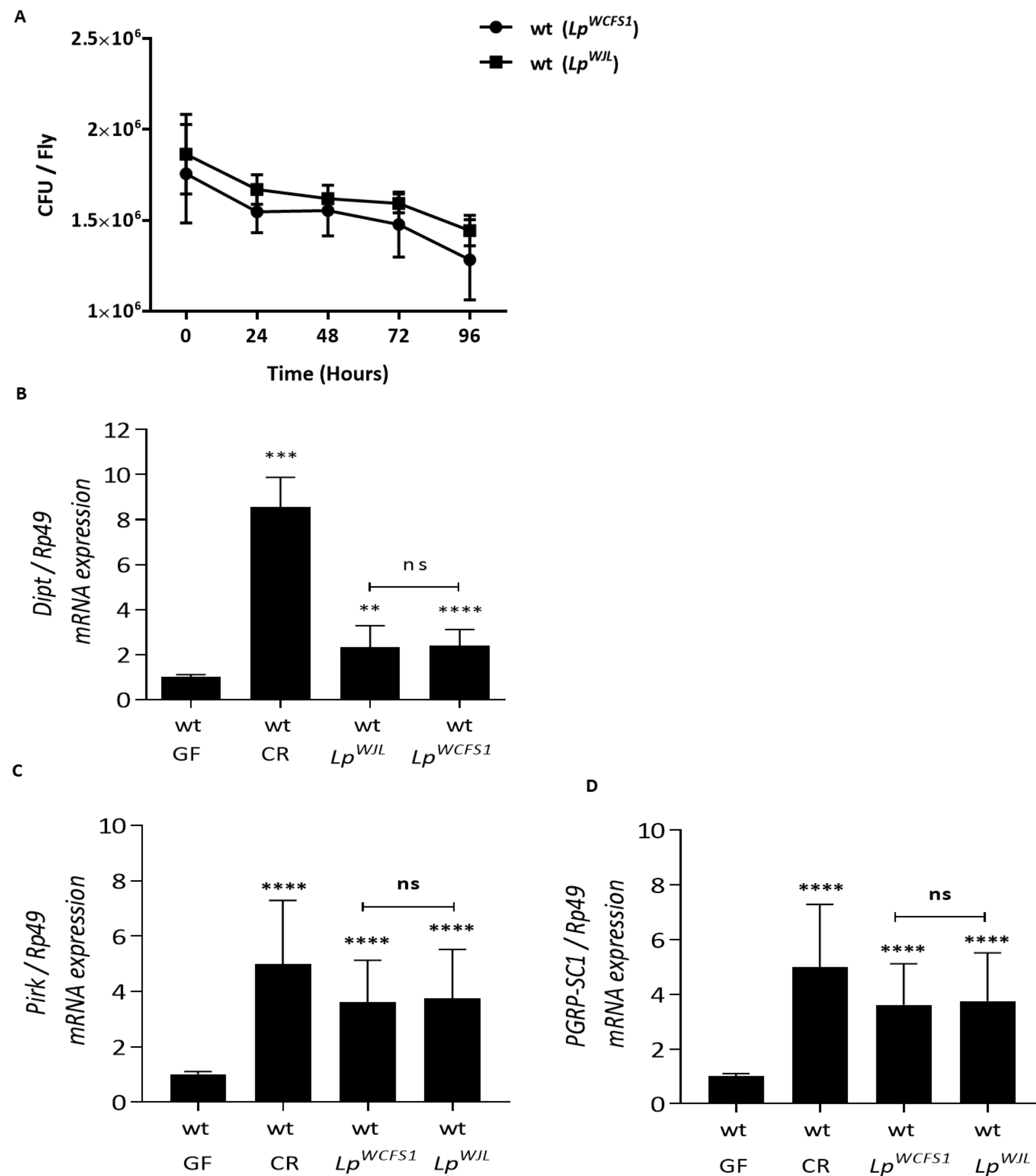


23

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

26 (A) Internal bacterial loads retrieved from the guts of Germ Free (GF) wild-type (wt) flies fed with *Lp*$^{WCFS1}$ or *Lp*$^{WJL}$
27 for three days. Colony Forming Unit (CFU) counting was performed every 24 hours after transferring the
28 monoassociated flies to a fresh cornmeal medium vial.
29 (B, C and D) Relative expression of the *Diptericin* (*Dipt*) (B) *Pirk* (C) and *PGRP-SC1* (D) transcripts in the midgut of
30 Germ Free (GF) wild-type (wt) flies fed with *Lp*$^{WCFS1}$ or *Lp*$^{WJL}$ for 3 days. Transcripts expression was measured by RT-
31 qPCR in total RNA extracts. Ribosomal protein 49 (Rp49) transcript was used as reference gene. Transcripts levels
32 detected in GF flies is set as a control.
33 Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were
34 performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$;
35 ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

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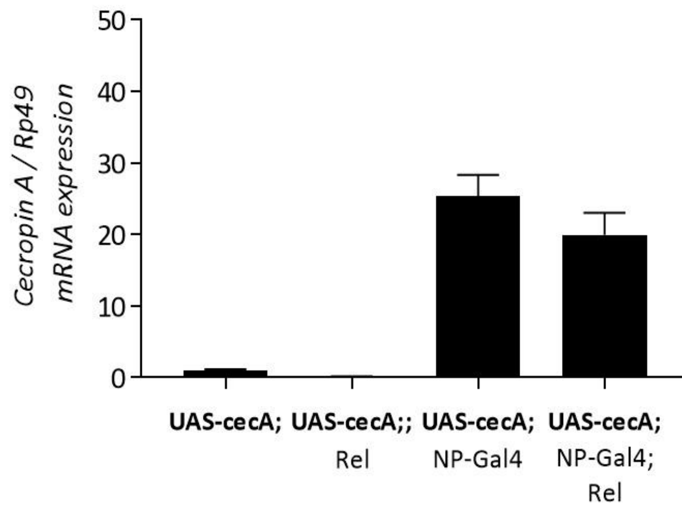


36

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

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

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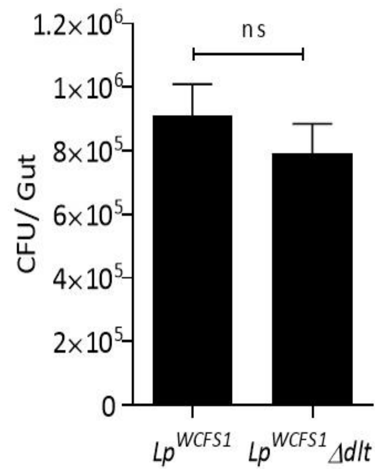


42

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

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

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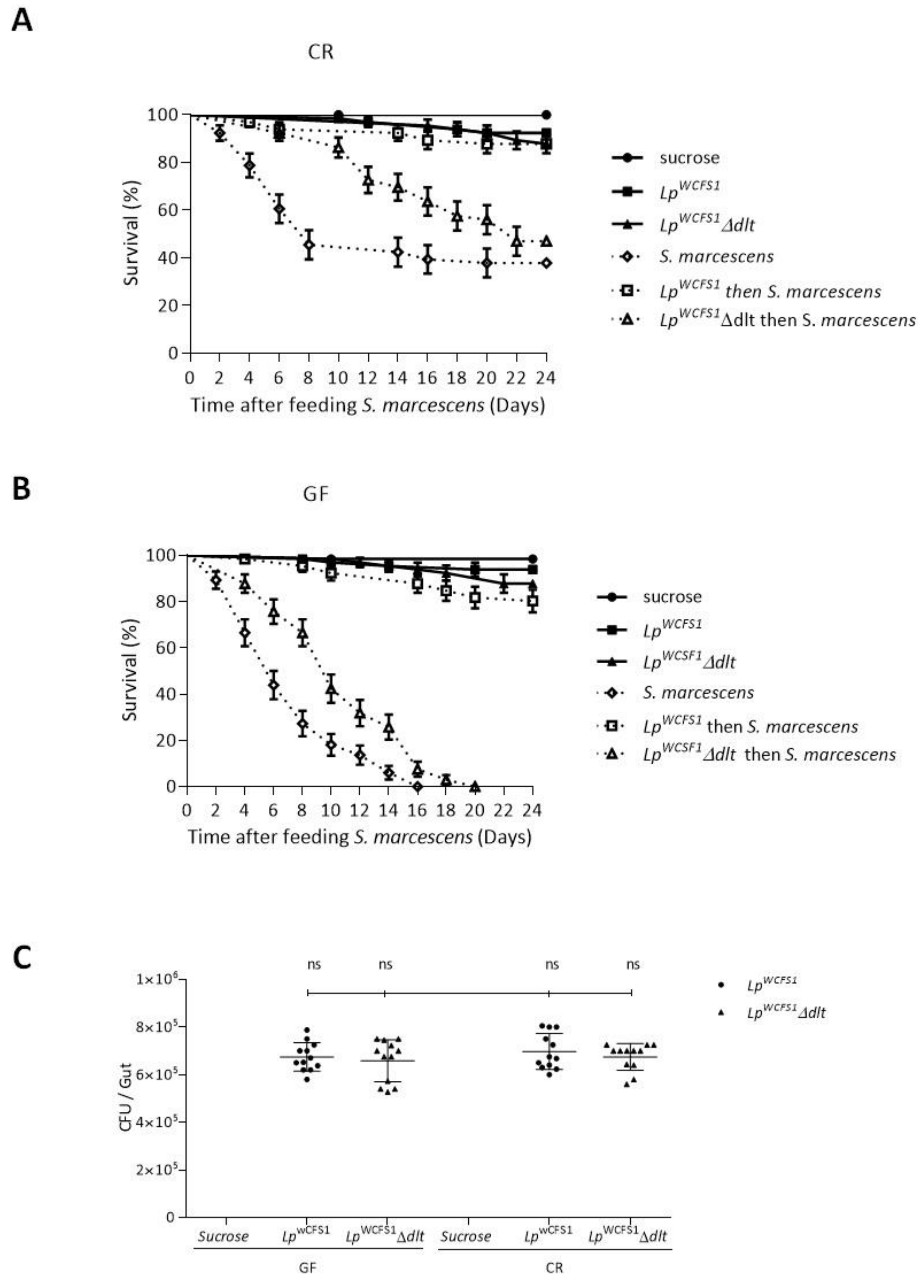


47

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

50 (A and B) Conventionally reared (CR) (A) and Germ Free (GF) (B) wt flies were fed with *Lp*$^{WCSF1}$ or *Lp* $^{WCFS1}$*Δdlt* for
51 3 days prior to *S. marcescens* challenge. Flies were fed a sucrose suspension of *S. marcescens* for 2 days and then
52 transferred to a fresh cornmeal vial.
53 (C) Internal bacterial loads retrieved from the guts of Germ Free (GF) or conventionally reared (CR) wt flies fed with
54 *Lp*$^{WCFS1}$ or *Lp* $^{WCFS1}$*Δdlt* for three days.
55 Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were
56 performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$).

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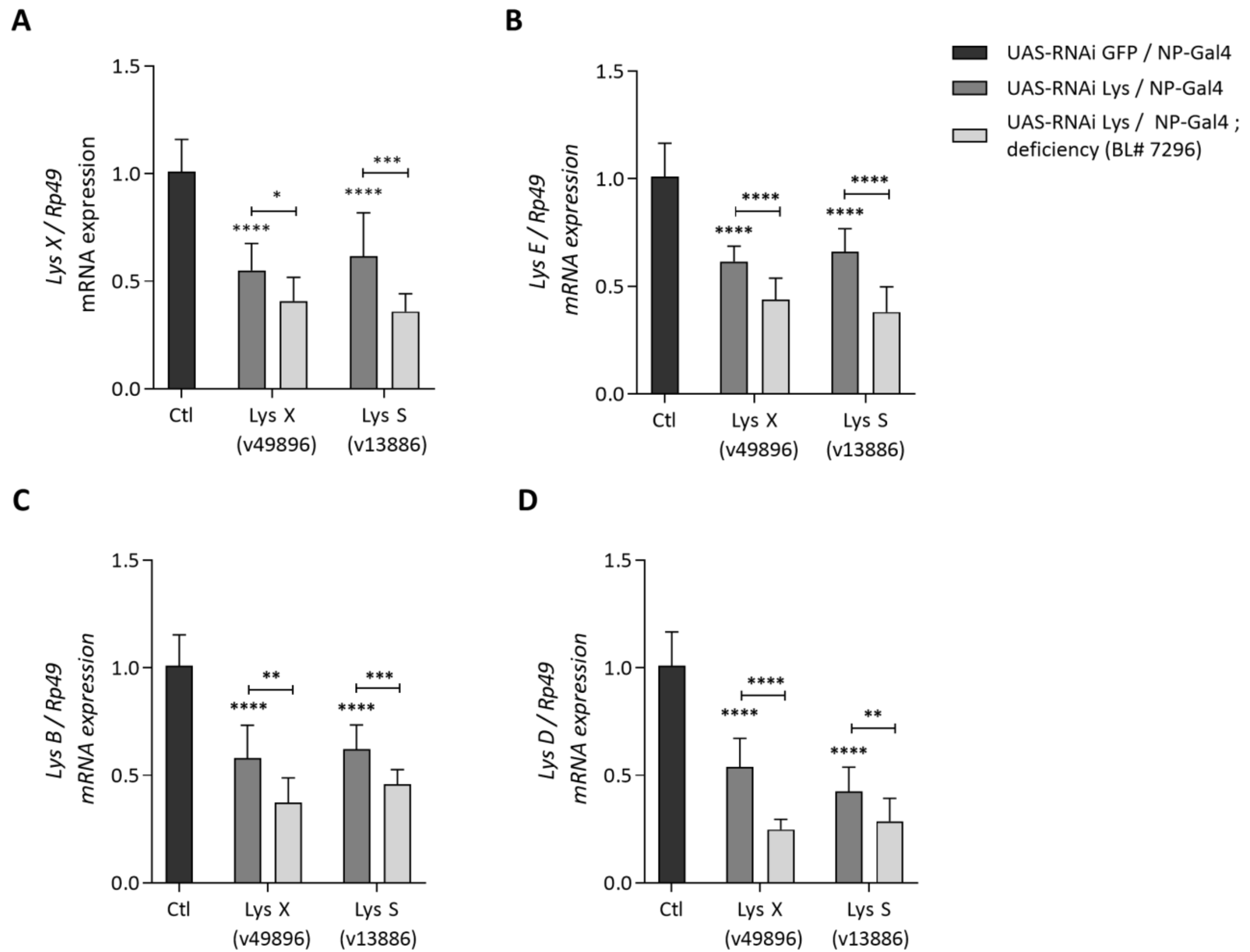


58

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

60 Silencing of the lysozyme genes was performed by the expression of RNAi constructs (UAS-RNAi) in the gut of
61 adult flies using the midgut specific driver NP-Gal4. Two different transgenic fly lines (v49896) and (v13886) were
62 used. These harbor different RNAi construct targeting the *LysX* or the *LysS* transcripts respectively. Silencing of
63 lysozyme genes is further aggravated by crossing these transgenic flies to a fly line carrying a chromosomal deletion
64 (deficiency BL#7296) uncovering the Lysozyme locus in the *Drosophila* genome. A transgenic line expressing an
65 RNAi construct targeting the GFP transcript is used as a control. Relative expression of the *Lysozymes X* (A), *E* (B),
66 *B*(C) and *D* (D) transcripts is measured in total RNA extracts from the midgut of the transgenic flies of the different
67 genetic background Ribosomal protein 49 (Rp49) transcript was used as reference gene. Transcripts levels are
68 compared to that detected in UAS-RNAi GFP transgenic flies that is arbitrary set to 1.
69 Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were
70 performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$;
71 ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).