D-Alanlyation of teichoic acids contributes to Lactobacillus plantarum-mediated Drosophila growth during chronic undernutrition

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The microbial environment influences animal physiology. However, the underlying molecular mechanisms of such functional interactions are largely undefined. Previously, we showed that during chronic undernutrition, strains of Lactobacillus plantarum, a major commensal partner of Drosophila, promote host juvenile growth and maturation partly through enhanced expression of intestinal peptidases. By screening a transposon insertion library of Lactobacillus plantarum in gnotobiotic Drosophila larvae, we identify a bacterial cell-wall-modifying machinery encoded by the pbpX2-dlt operon that is critical to enhance host digestive capabilities and promote animal growth and maturation. Deletion of this operon leads to bacterial cell wall alteration with a complete loss of d-alanlyation of teichoic acids. We show that L. plantarum cell walls bearing d-alanlyated teichoic acids are directly sensed by Drosophila enterocytes to ensure optimal intestinal peptidase expression and activity, juvenile growth and maturation during chronic undernutrition. We thus conclude that besides peptidoglycan, teichoic acid modifications participate in the host-commensal bacteria molecular dialogue occurring in the intestine.

Metazoa establish complex interactions with their resident microorganisms for mutual benefits1–3. When in homeostasis, these interactions contribute to different aspects of host physiology4–6. In the gut, microbial communities enhance digestive efficiency by providing enzymatic functions that help their hosts optimize extraction of dietary energy and nutrients. In addition, the gut microbiota promotes proper immune system development and local immune homeostasis, and limits pathogen colonization6–10. Despite the renewed interest in understanding the functional impact of gut microbiota on host physiology, a clear view of the molecular dialogue engaged during host–microbiota interaction remains elusive5,8,9. Therefore, the use of simple animal models, such as Drosophila, may help unravel the evolutionarily conserved mechanisms underlying the impact of intestinal bacteria in their host physiology, because it combines genetic and experimental tractability with a cultivable microbiota and the ease to generate germ-free (GF) animals11,12.

Drosophila gut microbiota is composed of simple and aero-tolerant bacterial communities (mostly Acetobacteraceae and Lactobacillaceae families) with five prevalent species: Acetobacter pomorum, Acetobacter tropicalis, Lactobacillus brevis, Lactobacillus plantarum and Lactobacillus fructivorans13,14. The genus Lactobacillus includes bacteria with high phylogenetic and functional diversity13,15. They have been largely used as model lactic acid bacteria and are recognized as potential health beneficial microorganisms in the human gastrointestinal tract17,18. As a prevalent member of Drosophila microbiota, L. plantarum is involved in several aspects of host physiology such as social behaviour15,19, protection against infection16,17, gut epithelial homeostasis11,21, nutrition11–23 and post-embryonic development1,2,13. We previously reported that, during chronic undernutrition, certain strains of L. plantarum (such as L. plantarum24, LpWJL) fully recapitulate the beneficial effect of a more complex microbiota by promoting Drosophila juvenile growth and maturation rate13, LpWJL exerts its beneficial effect on larval growth through the host nutrient-sensing system that relies on the tissue-specific activity of the TOR kinase, which subsequently modulates hormonal signals controlling growth and maturation25. Importantly, using conventional and gnotobiotic mice, we recently demonstrated that the intestinal microbiota and some strains of L. plantarum also influence linear growth in mammals14,15. These results suggest that the still unknown molecular mechanisms underlying microbiota-mediated juvenile growth promotion are probably conserved during evolution. Recently, we showed that L. plantarum influences juvenile growth at least partly through the increased expression of a set of specific host digestive enzymes in the intestine14,15. We have shown that LpWJL promotes the expression of peptidases, such as Jon66Ci and Jon66Cii, in the enterocytes in both a PGRP-LE/Imd/Relish-dependent and -independent manner. The resulting enhanced peptidase activity in the midgut increases the digestion of dietary proteins into dipeptides and amino acids as well as their uptake. Circulating dipeptides and amino acids are sensed in endocrine tissues by the TOR kinase pathway, which promotes the production of Drosophila growth factors, the insulin-like peptides and a precocious peak in the production of the moulting steroid hormone ecdysone16.

Here, we aim to identify the bacterial genetic determinants involved in L. plantarum-mediated juvenile growth promotion. Through the generation of a random transposon-mediated insertion

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library in the growth-promoting strain \textit{L. plantarum}NC8 (LpNC8), we identified a set of transposon insertions altering host growth promotion. Among these insertions, we further characterized the insertion in the \textit{pbpX2-dlt} operon with predicted functions in cell wall biogenesis and remodelling. Deletion of this operon alters the bacterial cell wall due to complete loss of \textit{d}-alanation of teichoic acids (TAs), and significantly impairs \textit{Drosophila} larval growth, maturation, and expression and activity of intestinal peptidases. Our analysis points to the existence of additional host sensing and signalling mechanisms, besides PGRP-LE/Imd/Relish, involved in the sensing of the cell wall features defined by the gene products of the \textit{pbpX2-dlt} operon. Taken together, our results demonstrate that \textit{d}-alan esterification of TAs directly contributes to \textit{L. plantarum}-mediated \textit{Drosophila} growth promotion during chronic undernutrition.

**Results**

**Generation of a \textit{L. plantarum}NC8 random-mutagenesis library.** To identify \textit{L. plantarum} genes required to sustain \textit{Drosophila} juvenile growth and maturation, we adopted a classical unbiased forward genetic approach using transposon-mediated mutagenesis of the bacterial chromosome coupled to a phenotypical screening in mono-colonized animals. The previously characterized strain \textit{LpGLV} has a low transformation efficiency (R.C.M., unpublished results) and carries plasmids \textit{pBI11}, \textit{pBI12}, \textit{pBI14} that preclude random transposition in the bacterial chromosome. We therefore chose a \textit{L. plantarum} strain with a high transformation efficiency, no native plasmids and capable of promoting host growth in mono-assocation experiments to the same extent as \textit{LpGLV}. This strain, designated as \textit{LpNC8}, is suitable for transposon mutagenesis library construction. Upon mono-colonization, both strains strongly support \textit{Drosophila}’s larval linear growth (Fig. 1a) and maturation (that is, entry to metamorphosis; Fig. 1b) under chronic undernutrition when compared with the moderate-growth-promoting strain \textit{L. plantarum}NIZO287\textsuperscript{1223} (Lp\textsuperscript{NIZO287\textsuperscript{1223}}, refs \textsuperscript{11,12,30}) or the GF condition (Fig. 1a,b). Thus, we mutagenized the \textit{LpNC8} chromosome using the \textit{Pm\textsuperscript{r}-TpS\textsuperscript{1223}} transposon mutagenesis system previously described in refs \textsuperscript{3,11}. This system was developed for lactic acid bacteria and has been successfully applied to \textit{Lactobacillus casei}\textsuperscript{335} and \textit{Lactobacillus pentosus}\textsuperscript{17}. It couples a thermo-sensitive plasmid (pVI1129) transiently expressing the \textit{Is\textsuperscript{1223}} transposase and a suicide plasmid (pVI1110) encoding the \textit{Is\textsuperscript{1223}} transposon, which together lead to random insertion of \textit{Is\textsuperscript{1223}} sequences into the bacterial chromosome. Strain NC8pVI1129 (Supplementary Table 1) was transformed with pVI1110 and 2,091 colonies were randomly selected and stocked as individual clones at \textdegree{}C. To evaluate the randomness of the transposon insertions in our library, we tracked transposon insertion sites by deep sequencing of flanking genomic sequences (see Methods). Sequencing reads were mapped to the \textit{LpNC8} genome, which revealed that transposon insertions are evenly distributed over the \textit{LpNC8} genome with an average insertion site every 2 kilobases (Fig. 1c). By analysing sequencing reads and insertion sites, we found that among the 2,091 insertions, 1,574 insertions disrupted 1,218 different open reading frames (ORFs; 42\% of \textit{LpNC8} ORFs currently annotated; Supplementary Table 2) and 517 landed in intergenic regions. Insertions were detected in ORFs belonging to different clusters of orthologous groups (Supplementary Table 2 and Supplementary Fig. 1) and, globally, only minimal differences in the relative proportion of functional groups targeted in our library were observed as compared with the repartition of clusters of orthologous groups in the \textit{LpNC8} genome (Supplementary Fig. 1). These results demonstrate that the insertion library sufficiently covers the genome in a random manner, thus making it suitable for further phenotypic screening.

**Screening of the \textit{LpNC8} mutant library identifies mutants affecting larval growth promotion.** We screened the insertions library with the aim of identifying \textit{LpNC8} mutants, which, following mono-colonization, have an altered capacity to sustain growth and maturation of chronically undernourished \textit{Drosophila}. Prior to the library screening, we experimentally determined that in these nutritional conditions, the most robust timing to visually discriminate (by counting the number of pupae emerging from food) a strong growth-promoting strain (Lp\textsuperscript{NC8\textsuperscript{1223}}), a moderate growth-promoting strain (Lp\textsuperscript{NIZO287\textsuperscript{1223}}; refs \textsuperscript{11,12}) and the GF condition is day 8 after inoculation of the GF eggs with bacterial isolates (Fig. 1b and Supplementary Fig. 2). We searched for \textit{LpNC8-pVI110} insertion mutants with a growth-promotion capacity weaker than or similar to Lp\textsuperscript{NIZO287\textsuperscript{1223}} (Fig. 1b and Supplementary Fig. 2). The screen was conducted as follows: 20 GF eggs were deposited in tubes containing low-yeast diet and were inoculated independently with each one of the 2,091 \textit{LpNC8-pVI110} insertion strains, while Lp\textsuperscript{NC8\textsuperscript{1223}} and GF served as controls. After 8 days of development, the number of emerging pupae was scored in each tube and normalized into \textit{z} scores (that is, the score for each strain reflecting the number of standard deviations from the mean number of pupae scored at day 8 from all tested strains; mean = 15.18 pupae, s.d. = 3.33; Fig. 1d). The control strains yielded \textit{z} scores of −0.05 for \textit{LpNC8} and −3.73 for Lp\textsuperscript{NIZO287\textsuperscript{1223}}. This screen revealed insertions leading to either gain- or loss-of-function phenotypes as compared with the reference strain \textit{LpNC8}, with \textit{z} scores ranging from +2.73 down to −4.52 (Fig. 1d). To select robust loss-of-function candidates similar to or stronger than the Lp\textsuperscript{NIZO287\textsuperscript{1223}} strain, we set a selection cutoff for \textit{z} scores below −3 and identified 28 transposon insertion mutants, which were retained for a secondary screening procedure. These candidates were re-tested in three independent experiments as during the primary screen but this time the number of emerging pupae was recorded at days 7, 8 and 9. From the secondary screen, we confirmed that seven insertion mutants robustly delayed pupariation time with statistical significance when compared with the wide-type (WT) \textit{LpNC8} strain and were statistically undistinguishable from the Lp\textsuperscript{NIZO287\textsuperscript{1223}} strain (Fig. 1e,f). To exclude the possibility that the loss of the growth-promoting phenotype of these seven mutants was a consequence of their inability to persist in the low-yeast fly food, we assessed bacterial loads in the food matrix at days 3, 5, 7 and 10 after initial inoculation of 10\textdegree colony-forming units (CFUs) ml\textsuperscript{−1} for each strain (Supplementary Fig. 3). All of the mutant strains behave in the same way as the \textit{LpNC8} strain at each time point.

Having characterized the impact of the seven insertion mutants on \textit{Drosophila} growth promotion, we identified the transposon insertion sites in the \textit{LpNC8} genome. Of the seven insertions, six were inside ORFs and one in an intergenic region between the ORFs encoding the transporter \textit{secE} and the transcription regulator \textit{mesG} (Table 1 and Supplementary Fig. 4). The insertions in ORFs hit the \textit{mleI} gene encoding a malate transport protein (referred to as \textit{1-74}), the \textit{pbpX2} gene encoding a putative serine-type \textit{d-d}-carboxypeptidase (referred to as \textit{6-20}), the \textit{pts28ABC} gene encoding a PTS system component (referred to as \textit{14-10}), the \textit{dnaK} gene encoding a chaperone protein (referred to as \textit{22-19}) (Table 1). Previously, we reported that peptidoglycan (PG) recognition by PGRP-LE partly contributes to \textit{LpNC8}-mediated enhanced expression of intestinal peptidase during juvenile growth promotion\textsuperscript{3,11}. Hence, we further characterized the 6-20 mutant, hitting \textit{pbpX2}, with a predicted function in PG biosynthesis/maturation\textsuperscript{1,12,13}.

**Deletion of the \textit{pbpX2-dlt} operon affects \textit{LpNC8}-mediated larval growth promotion.** To confirm the loss-of-function phenotype observed with the transposon insertion mutant 6-20, we generated a deletion mutant of the \textit{pbpX2} gene in the \textit{LpNC8} strain by
Fig. 1 | Identification of L. plantarumNC8 loci involved in Drosophila growth promotion. a, Larval longitudinal length after inoculation with LpWJL (n = 56), LpNC8 (n = 60) and LpNIZO2877 (n = 37) strains and PBS (n = 60) for the GF condition. Larvae were collected seven days after association and measured as described in the Methods. The asterisks represent a statistically significant difference compared with the larval size of LpNIZO2877; NS represents the absence of a statistically significant difference between LpWJL and LpNC8 larval sizes. ****P < 0.0001. The horizontal bars in the graph represent means and s.d. A representative graph from one out of three independent experiments is shown. b, Number of emerged pupae scored over time from eggs associated with the strains LpWJL, LpNC8 and LpNIZO2877 or PBS (for the GF condition). Forty GF eggs were associated independently with 10⁶ CFUs of each one of the strains in five replicates. The number of pupae was scored every 24 h. c, Genome atlas of transposon insertions mapped to the LpNC8 genome. The innermost rings represent the GC skew (Circle 1) in purple/green and the GC content (Circle 2) in black. Circle 3 shows the distribution of the genomic regions disrupted by each transposon insertion (red bars). Circles 4 and 5 show LpNC8 coding regions (blue arrows), tRNAs (red arrows) and rRNAs (purple arrows) on the negative and positive strand respectively. d, Screen of the random transposon insertion library for mutants with an altered growth-promotion phenotype under chronic undernutrition. The numbers of pupae scored at day 8 after association with the 2,091 insertional mutants were converted to z scores. ****P < 0.0001; ***0.0001 < P < 0.001; **0.001 < P < 0.01. The insertions on the left of the dotted line were retained for further analyses. e, Number of emerged pupae 8 days after association of GF eggs with the 28 insertions selected from the primary screen after setting the z score to −3. Each strain was tested in five replicates. The bars in the graph represent means and the error bars represent s.d. A representative graph from one out of three independent experiments is shown. The purple asterisks represent a statistically significant difference compared with the LpNC8 number of pupae; NS represents the absence of a statistically significant difference compared to LpNC8 (purple) and LpNIZO2877 (blue). ****P < 0.0001; ***0.0001 < P < 0.001; **0.001 < P < 0.01. f, Number of emerged pupae at days 7, 8 and 9 after association of 20 GF eggs with 10⁶ CFUs of the seven insertions selected from the secondary screen. Each strain was tested in five replicates. A representative graph from one out of three independent experiments is shown. For exact P values, see Supplementary Table 4.
We did not detect a significant difference between the ability of peliosis and developmental timing assays and observed that \( \Delta \)dltXABCD Drosophila operon in LpNC8 molecular dialogue engaged between these symbiotic partners. Previously, inactivation of the operon encoding L. plantarumWCFS1 the strain \( \Delta \)lp_0594 was associated with reduced cell viability. However, while studying CFU counts, we did not detect any significant differences in bacterial cell doubling time. We therefore concluded that the deletion of the \( \Delta \)dltXABCD operon (and not just \( \Delta \)pbpX2) recapitulated the 6-20 mutant phenotype, showing an increased loss-of-function phenotype as \( \Delta \)dltop cells, yet, in contrast to those strains, the change in cell shape of the \( \Delta \)dltXABCD-dltXABCD mutant is not associated with reduced cell viability.

Next, we determined the amount of d-alanine esterified to TAs in \( \Delta \)pbpX2 and \( \Delta \)dltXABCD strains. d-Alanine was released from dried bacteria by mild alkaline hydrolysis and was quantified by high-performance liquid chromatography (Fig. 3d,e) as described previously\(^{27,32}\), allowing one to estimate d-alanine esterified to both LTAs and wall TAs (WTAs). d-Alanine was released in appreciable amounts from \( \Delta \)pbpX2 cells but was almost undetectable from the \( \Delta \)dltXABCD cells (Fig. 3d,e). We therefore conclude that the deletion of the pbpX2-dlt operon leads to a loss of d-alanine esterification of TAs and width reduction in the mutant cells. Thus, these observations indicate that d-alanine esterification of TAs is required for \( \Delta \)pbpX2-mediated Drosophila growth promotion during chronic undernutrition.

**pbpX2-dlt-dependent cell wall modification directly influences larval growth.** Altering the cell wall may have pleiotropic effects on bacterial cell physiology\(^{21}\). We therefore wondered whether the identified pbpX2-dlt-dependent cell wall modification influences larval growth directly (for example, through direct host sensing and signalling of cell wall motifs) or indirectly (for example, by indirectly altering the production of other functional compounds/metabolites by the mutant bacteria). To this end, we treated GF, and \( \Delta \)dltXABCD and \( \Delta \)pbpX2-dlt-associated animals daily with cell walls purified from either \( \Delta \)pbpX2 or \( \Delta \)dltXABCD cells (Fig. 3f). We observed that none of the treatments with purified cell walls triggers enhanced growth of GF larvae. However, treatments with cell walls purified from \( \Delta \)pbpX2 but not from \( \Delta \)dltXABCD cells could rescue the altered growth of \( \Delta \)dltXABCD-associated animals whereas they did not influence growth of \( \Delta \)pbpX2-associated animals. These results establish that purified cell walls on their own are not sufficient to trigger enhanced larval growth in GF animals but demonstrate that pbpX2-dlt dependent cell wall modification is necessary and sufficient to directly influence growth of L. plantarum-associated animals.

**Deletion of the pbpX2-dlt operon in LpNC8 affects cell morphology and d-alanylation of TA, but not bacterial growth dynamics.** Previously, inactivation of the operon encoding dlt genes in the strain L. plantarumWCFS1 (LpNC8) and the strain Lactobacillus rhamnosusGC (LrGC) had been associated with a major reduction of esterification of lipoteichoic acids (LTAs) by d-alanines, reduced bacterial growth rate and increased cell lysis\(^{1,2,28}\). Therefore, we investigated \( \Delta \)lptB mutant in the LpWCFS1 strain\(^{21,23}\), the \( \Delta \)dltXABCD operon enters the stationary phase with a lower OD\(_{600}\) value. While, however, studying CFU counts, we did not detect any significant differences in bacterial cell doubling time (Supplementary Fig. 5; 98 minutes for \( \Delta \)lptB versus 94 minutes for \( \Delta \)dltXABCD). This is in line with our previous results posing that the 6-20 mutant bacteria persist in the low-yeast fly food similarly to WT LpNC8 cells (Supplementary Fig. 3) and that \( \Delta \)dltXABCD mutant cells colonize larvae similarly to \( \Delta \)lptB cells (Fig. 2e). This striking difference between CFU counts and absorbance at 600 nm for the two strains prompted us to study the cell morphology of the LpNC8 and \( \Delta \)dltXABCD strains by phase-contrast and fluorescence microscopy following membrane staining with FM4-64 (Fig. 3a). We detected a significant reduction in the width of \( \Delta \)dltXABCD mutant cells (Fig. 3a; LpNC8 mean width of 783 nm ± 105; \( \Delta \)dltXABCD mean width of 658 nm ± 104). Scanning and transmission electron microscopy confirmed the reduced bacterial mid-cell width of \( \Delta \)dltXABCD versus LpNC8 cells (Fig. 3b,c). These observations are reminiscent of the dlt genes’ loss-of-function phenotypes seen in LpWCFS1 and LrGC; yet, in contrast to those strains, the change in cell shape of the \( \Delta \)dltXABCD-dltXABCD mutant is not associated with reduced cell viability.

**Table 1** | Loci of the insertions selected in the screen

| Mutant number | Locus of pV110 disruption | Annotation |
|----------------|---------------------------|------------|
| 74             | lp_0594                   | Malate transport protein (mleP) |
| 6-20           | lp_2021                   | Serine-type d-alanyl-d-alanyl carboxypeptidase (pbpX2) |
| 14-9           | IG lp0616-lp0617          | Intergenic region between seC and nusG |
| 14-10          | lp_3240                   | PTS system, β-glucosides-specific EIABC component (pts28ABC) |
| 17-57          | lp_2466                   | Prophage P2 protein 15, terminase large subunit |
| 18-65          | lp_1944                   | Multidrug ABC transporter |
| 22-19          | lp_2027                   | Chaperone, heat-shock protein DnaK |
postulated that, in addition to PG sensing by PGRP-LE followed by Imd/Relish signalling, additional compounds produced by the bacteria would affect intestinal peptidase expression and activity through yet unknown mechanisms. Given that the Δdlttop strain is a poor Drosophila growth-promoting strain with an altered cell wall composition, we hypothesize that the pbpX2-dlt operon affects Drosophila growth. The pbpX2-dlt operon genomic organization in the LpNC8 strain: pbpX2/dltX/dltA/dltB/dltC/dltD. 6-20, pVI110 insertion within pbpX2 gene. ΔpbpX2 mutant, in which pbpX2 gene was deleted by homology-based recombination. ΔdltXABCD mutant, in which dltXABCD genes were deleted by homology-based recombination. Δdlttop mutant, with genes pbpX2/dltX/dltA/dltB/dltC/dltD deleted by homology-based recombination. pbpX2 gene was deleted by homology-based recombination.

To test this hypothesis, we investigated how the Δdlttop strain influences intestinal peptidase expression and activity. To this end, we inoculated GF eggs with either sterile PBS, LpNVCA or Δdlttop strains, collected size-matched larvae at mid-L3 stage, dissected their midguts and assayed intestinal peptidase activity and expression (Fig. 4a). We detected increased peptidase activity in the midgut of LpNVCA-associated animals as compared with GF midguts and this induction was diminished in Δdlttop-associated animals (Fig. 4b). Accordingly, among the intestinal peptidase genes previously reported to be induced following L. plantarum association, two (Jon66Ci and Jon65Ai) show a significantly reduced induction in Δdlttop-associated animals as compared with LpNVCA-associated animals (Fig. 4c and Supplementary Fig. 6a). Given the direct influence of pbpX2-dlt-dependent cell wall modification on larvae growth, we tested whether purified cell wall would trigger intestinal peptidase expression. To this end, we acutely treated GF animals at mid-L3 with cell wall purified from either LpNVCA or Δdlttop cells, and assayed intestinal peptidase gene expression in dissected midguts (Fig. 4d). At 6 hours post-inoculation, cell walls purified from either LpNVCA or Δdlttop cells now triggered increased expression of Jon66Ci albeit to a small extent. At 24 hours post-inoculation, cell walls purified from Δdlttop cells now triggered increased expression of Jon66Ci albeit to a small extent.
significantly reduced level than following \( \text{L} p^{\text{NC8}} \) cell wall treatment. Of note, induction of the \( \text{Jon66Cii} \) and \( \text{Jon65Ai} \) genes following acute treatment with purified cell wall or live cells did not fully recapitulate the extent of gene induction observed on chronic treatment with live cells (Supplementary Fig. 6); however, the host transcriptional response to acute treatment with purified cell wall is very similar to the response to acute treatment with live cells (Supplementary Fig. 6d). Taken together, these results establish that \( \text{Drosophila} \) enterocytes directly sense cell walls bearing \( \text{ppbX2-dlt} \) dependent \( \text{d}-\text{alanine} \) esterification of TA, a signal that selectively triggers intestinal peptidase induction.

Sensing of multiple cell wall motifs is required for \( \text{L} p^{\text{NC8}} \) mediated intetestinal peptidase expression and larval growth promotion. Next, we tested whether the impaired induction of intestinal peptidases by \( \Delta \text{dlt}_{op} \) cells and purified cell wall is further altered in \( \text{Dredd} \) larvae. \( \text{Dredd} \) encodes an essential positive regulator of the PG-sensing Imd/Relish cascade\(^{26,28,29} \). In \( \text{Dredd} \) larvae, the PGRP-LE/Imd/Relish signalling cascade is completely inactivated in the enterocytes following \( \text{L} p^{\text{NC8}} \) association\(^{3,23,26} \). We confirmed this observation by analysing the expression of several Imd/Relish target genes (PGRP-SC1, \( \text{attD} \), PGRP-LB and \( \text{Pirk} \)) whose expression in the midgut entirely relies on the PGRP-LE/Imd/Relish cascade following \( \text{L} p^{\text{NC8}} \) association\(^{26,28} \). We found that while PGRP-SC1, \( \text{attD} \), PGRP-LB and \( \text{Pirk} \) failed to be induced by either cells or purified cell wall from \( \text{L} p^{\text{NC8}} \) or \( \Delta \text{dlt}_{op} \) mutant in \( \text{Dredd} \) larvae, both bacterial strains and purified cell walls readily activated their expression in control larvae with no difference between the two bacterial strains (Supplementary Fig. 6bc). These results indicate that the \( \Delta \text{dlt}_{op} \) deletion does not alter the Dredd-dependent sensing of \( \text{L} p^{\text{NC8}} \) PG by the WT host. This result suggests that the effect mediated through \( \text{L} p^{\text{NC8}} \) cell walls bearing \( \text{d}-\text{alanylated} \) TA activates another commensal-sensing and signalling pathway in enterocytes in addition to the PGRP-LE/Imd/Relish cascade, which probably directly senses \( \text{d}-\text{alanylated} \) TA.

Next, we wished to examine whether this second commensal-sensing host pathway genetically interacts with the Dredd-dependent PG-sensing pathway, and how this interaction affects the expression of intestinal peptidases in response to \( \text{L} p^{\text{NC8}} \). We analysed the expression of the intestinal peptidases in dissected midguts from \( \text{Dredd} \) larvae mono-associated with either \( \text{L} p^{\text{NC8}} \) or \( \Delta \text{dlt}_{op} \) cells (Fig. 4c and Supplementary Fig. 6a) or acutely inoculated with their purified cell walls (Fig. 4de). In contrast to control larvae, where only \( \text{Jon66Cii} \) and \( \text{Jon65Ai} \) expression levels were altered, the expression levels of \( \text{Jon66Cii} \), \( \text{Jon65Ai} \), \( \text{CG18179} \), \( \text{CG18180} \), \( \text{Jon99Ci} \) and \( \text{Jon44E} \) peptidases in \( \text{Dredd} \) mutants were strongly

**Fig. 3** Cell envelope changes related to \( \text{ppbX2-dlt} \) operon deletion. a, Left: observation of \( \text{L} p^{\text{NC8}} \) (n = 489) and \( \Delta \text{dlt}_{op} \) (n = 542) cells by phase-contrast microscopy following membrane staining with FM4-64. Representative images from one out of three independent experiments are shown. b, Left: observation of \( \text{L} p^{\text{NC8}} \) (n = 159) and \( \Delta \text{dlt}_{op} \) (n = 153) cells by scanning electron microscopy. c, Left: observation of \( \text{L} p^{\text{NC8}} \) (n = 20) and \( \Delta \text{dlt}_{op} \) (n = 21) cells by transmission electron microscopy. The right panels in a−c show \( \text{L} p^{\text{NC8}} \) and \( \Delta \text{dlt}_{op} \) cell width measurements from each microscopy observation. The bars in the graphs represent means and s.d. Cellular eluted compounds were detected by ultraviolet absorbance at 340 nm. Quantification was achieved by comparison with \( \text{PGRP-SC1} \) Imd/Relish target genes (\( \text{Jon66Cii} \), \( \text{Jon65Ai} \), \( \text{CG18179} \), \( \text{CG18180} \), \( \text{Jon99Ci} \) and \( \text{Jon44E} \) peptidases in \( \text{Dredd} \) mutants were strongly

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**ARTICLES**

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**Fig. 4 | Drosophila-reduced protease expression in the presence of the Δdlt<sup>op</sup> strain is independent of the Imd pathway.**

**a**, Experimental set-up for proteolytic activity assessment and RT-qPCR analysis. Forty germ-free eggs of y, w and y, w,Dredd flies were inoculated with 10<sup>7</sup> CFUs of Lp<sup>nc8</sup> or Δdlt<sup>op</sup> strains. For each condition, size-matched larvae were collected at the mid-L3 phase and their guts were dissected for proteolytic activity determination and RNA extraction followed by RT-qPCR. **b**, Proteolytic activity of dissected guts associated with Lp<sup>nc8</sup> or Δdlt<sup>op</sup> strains. The central bars in the graph represent means. A representative graph from one out of three independent experiments is shown. The purple asterisks represent a statistically significant difference compared with the GF condition. The green asterisks represent the absence of a statistically significant difference compared to the GF condition, and the purple NS represents the absence of a statistically significant difference compared to GF guts proteolytic activity. **0.001 < P < 0.01, U, enzymatic units.**

**c**, Mean ± s.d. of ΔCT<sup>protease</sup>/ΔCT<sup>protease</sup> ratios for Jon66Ci<sup><i>i</i></sup> and Jon65Ai detected in dissected guts associated with Lp<sup>nc8</sup> and Δdlt<sup>op</sup> or the GF condition from five biological replicates. Representative graphs from one out of three independent experiments are shown. The purple asterisks represent a statistically significant difference compared with Lp<sup>nc8</sup> protease expression. The green asterisks represent a statistically significant difference compared with the GF condition. NS represents the absence of a statistically significant difference compared to the GF condition. **0.001 < P < 0.01, *P < 0.05.**

**d**, Experimental set-up to assess the direct sensing of bacterial cell wall (CW) by larvae. Forty germ-free eggs of y, w and y, w,Dredd flies were kept axenic until they reached mid-L3 stage (ten days after GF egg collection), when they were inoculated with purified cell walls from Lp<sup>nc8</sup> or Δdlt<sup>op</sup> strains. For each condition, larvae were collected at 6 or 24 hours after association and their guts were dissected for RNA extraction followed by RT-qPCR. **e**, Mean ± s.d. of ΔCT<sup>protease</sup>/ΔCT<sup>protease</sup> ratios for Jon66Ci<sup><i>i</i></sup> and Jon65Ai detected in guts of axenic larvae inoculated with purified cell walls from Lp<sup>nc8</sup> and Δdlt<sup>op</sup> or the GF condition from five biological replicates. The central bars in the graph represent means. Representative graphs from one out of three independent experiments are shown. The purple asterisks represent a statistically significant difference compared with Lp<sup>nc8</sup> protease expression. The green asterisks illustrate a statistically significant difference compared with the GF condition. The green NS represents the absence of a statistically significant difference compared to the GF condition, and the purple NS compared with Lp<sup>nc8</sup>. **0.001 < P < 0.01; *P < 0.05.** For exact P values, see Supplementary Table 4.
altered following Δ\textit{dlt} operon mutation or inoculation with its purified cell walls as compared with the \textit{LpXO} condition.

This result, combined with our expression study of the PGRP-LE/Imd/Relish targets (Supplementary Fig. 6b,c), illustrates that the mechanisms sensing cell walls bearing \textit{d}-alanylated TA act in concert with Dredd-dependent PG sensing to elicit optimal expression response from intestinal peptidases loci. In the presence of the Δ\textit{dlt} operon mutation, the host mechanisms sensing cell walls bearing \textit{d}-alanylated TA are impaired, and the \textit{Dredd} mutation exacerbates the Δ\textit{dlt} operon-related host response, which culminates to diminish intestinal peptidase expression. Since the Δ\textit{dlt} operon mutation abolishes the \textit{d}-alanine esterification of bacterial TA, our results indicate that the host enterocytes sense and signal the presence of \textit{L. plantarum} cells by at least two mechanisms: through PGRP-LE-mediated PG fragment recognition and Imd/Relish signalling; and sensing of \textit{d}-alanylated TA and signalling by yet-to-be-discovered host mechanisms (Fig. 5d).

**Sensing of multiple cell wall motifs is required for \textit{LpXO}-mediated larval growth promotion.** Finally, we probed the consequence on \textit{L. plantarum}-mediated larval growth promotion of altering both bacterial signals. We previously showed that altering Dredd-dependent PG sensing in the enterocytes was not sufficient to alter \textit{L. plantarum}-mediated \textit{Drosophila} growth promotion\(^\text{6,10,13}\). Here we compared the larval growth and maturation of control and \textit{Dredd} animals following association with either the \textit{LpXO} or Δ\textit{dlt} operon strains. We observed a significant effect on both larval growth (Fig. 5a) and maturation (Fig. 5b,c) of coupling the Δ\textit{dlt} operon bacterial genotype and the host \textit{Dredd} genotype. Taken together, these results demonstrate that the host machineries in enterocytes sensing and signalling bacterial PG and cell walls with \textit{d}-alanylated TA are required for optimal \textit{L. plantarum}-mediated \textit{Drosophila} growth maturation and during chronic undernutrition (Fig. 5d).

**Discussion**

The results obtained in this study identify the \textit{L. plantarum} \textit{phpX2-dlt} operon as encoding an important bacterial functionality required to sustain a host–commensal dialogue that is beneficial to the host. Deletion of the \textit{phpX2-dlxXABCD} genes results in impairment of \textit{L. plantarum}-mediated \textit{Drosophila} gut peptidase expression and activity, larval growth and maturation. We show that the \textit{phpX2-dlt} operon is a key genetic determinant shaping \textit{L. plantarum} cell wall and cell shape via \textit{d}-alanine esterification of TAs. Moreover, we reveal that these changes in the cell envelope architecture and composition are critical for bacterial cell wall sensing and signalling by \textit{Drosophila} enterocytes, which underlies the beneficial interaction between \textit{Drosophila} and its symbiont \textit{L. plantarum} leading to improved growth during chronic undernutrition.

Despite \textit{L. plantarum} being a model lactic acid bacteria, random transposon mutagenesis is difficult to achieve due to low transformation efficiencies and/or instability of the transposon-delivering vector\(^\text{1,2}\). Here, we successfully employed the \textit{P\textit{trans}}-\textit{TaseIS} \textit{S22} system\(^\text{1,2,29}\) by constructing a random transposon mutant library in \textit{LpXO}. We collected and screened 2,091 transposon insertion mutants covering 1,218 genes and tested around 42% of the predicted protein-coding sequences in the \textit{LpXO} genome\(^\text{1,4}\). By increasing the number of transposon insertion mutants, one could target virtually all non-essential genes. On screening of our library for its ability to promote larval growth after association with each one of the insertion mutants, we identified seven insertions that severely impaired the \textit{L. plantarum} growth-promotion phenotype. Although significantly different from \textit{LpXO}, none of the insertions reflects a complete loss-of-function phenotype that completely mimics the GF condition. This observation suggests that the \textit{L. plantarum} growth promotion effect is probably multifactorial and to achieve such a ‘GF-like’ phenotype, we would need to target multiple bacterial functions.

Among the seven insertions, we were particularly interested by the one affecting the \textit{phpX2-dlt} operon, which is involved in \textit{L. plantarum} cell wall biogenesis and remodelling, and whose deletion significantly impaired \textit{Drosophila} larval growth and interfered with host peptidase expression. Cell wall structure, composition and organization play major roles in host–bacteria dialogue as they represent the core bacteria components that trigger the initial microbe-sensing response in the host. \textit{phpX2} encodes a putative \textit{d}-carboxypeptidase with homology to various low-molecular-weight penicillin-binding proteins\(^\text{1,2,29}\). The presence of \textit{phpX2} upstream of the \textit{dlt} genes is a unique feature of \textit{L. plantarum} genomes\(^\text{1,2,3}\), but the physiological role of \textit{phpX2} and the importance of the genetic linkage between \textit{phpX2} and the \textit{dlt} genes remain unknown. The \textit{dlt} genes are well described in several Gram-positive bacteria as being responsible for the esterification of TAs with \textit{d}-alanine\(^\text{6,10,13}\). TAs are anionic polymers localized within the Gram-positive bacteria cell wall, representing up to 50% of the cell envelope dry weight\(^\text{1,2,26}\). There are two types of TA: WTAs, which are covalently bound to PG, and LTAs, which are anchored on the cytoplasmic membrane\(^\text{5,21}\). The current model states that initially DltA ligates \textit{d}-alanine onto the carrier protein DltC. With the help of DltB, \textit{d}-alanine is then transferred from DltC to undecaprenyl phosphate, then crossing the membrane, where DltD transfers the \textit{d}-alanine to LTA\(^\text{5,21}\). Although still not fully understood, it seems that \textit{d}-alanyl-LTA serves as a donor for \textit{d}-alanine substitutions in WTA\(^\text{11}\). Despite being encoded upstream of \textit{dlt} in several Gram-positive bacteria, the role of DltX in \textit{TAR} \textit{d}-alanylation remains unknown. \textit{LpXO} carries its genome the genetic information to produce both types of TA\(^\text{1,26}\). Attachment of \textit{d}-alanine substituents to these structures is an important mechanism by which bacteria modulate surface charge, whose level has a major impact on TA functionalities, such as the control of autolysis\(^\text{3}\), maintenance of cell wall morphology\(^\text{3}\) and signalling with cells of their animal host\(^\text{34,36,41}\). \textit{L. plantarum} strains lacking \textit{d}-alanine substitutions on their TAs reduce secretion of proinflammatory cytokines but better stimulate IL-10 production in peripheral blood monocytes when compared with its WT, showing the importance of \textit{TA} \textit{d}-alanylation in immunomodulating properties in mammals\(^\text{14,15,18}\). To our knowledge, direct sensing of TA and downstream signalling events have never been reported in \textit{Drosophila}, yet \textit{d}-alanylation of WTA seems an important bacterial feature affecting immunomodulation because its hampers recognition of \textit{Staphylococcus aureus} PG by the pattern recognition receptor PGRP-SA\(^\text{36}\).

By quantification of \textit{d}-alanine released from whole cells, we determined that the \textit{phpX2-dlt} operon is responsible for \textit{d}-alaninylation of \textit{TA} in \textit{LpXO}. Moreover, the absence of the \textit{phpX2-dlt} operon affects cell morphology in \textit{LpXO} while cell viability and growth dynamics are not affected, in contrast to other lactobacilli strains\(^\text{1,2,26}\). Therefore, the consequences of deleting \textit{dlt} genes in lactobacilli are strain dependent. This study highlights the role of \textit{phpX2-dlxXABCD} genes and their impact on \textit{TAR} \textit{d}-alanine esterification for a bacterial beneficial effect on its host, a feature that is traditionally associated with pathogenesis and antibiotic resistance of Gram-positive bacteria\(^\text{11,30}\). Our results strengthen the role of \textit{TAR} modifications on commensal–host interactions and pave the way to further studies aiming at pinpointing the effect of \textit{phpX2-dlt} function on \textit{L. plantarum} cell biology and physiology as well as probing the physiological role of \textit{phpX2}.

We previously established that the promotion of \textit{Drosophila} linear growth by \textit{L. plantarum} is partly dependent on increased expression of host peptidases. We also demonstrated that such increased peptidase expression is partly controlled by the PGRP-LE/Imd/Relish signalling cascade\(^\text{1}\), a signalling pathway triggered by DAP-containing PG fragments\(^\text{29}\). Here we show that \textit{phpX2-dlt} dependent modification of bacterial cell walls also contributes to host intestinal peptidase induction and \textit{Drosophila} larval growth in parallel.
In this study, in WT hosts we observed reduced peptidase expression and activity following association with the Δdltop strain when compared with the WT L. plantarum strain. This differential peptidase expression is exacerbated in animals impaired in Imd/Relish signalling. Specifically, in Dredd mutants, the peptidase expression level following Δdltop strain association or treatment with Δdltop purified cell walls is close to that of the GF condition. Moreover, low peptidase expression following Δdltop in Dredd mutants correlates with poor larval growth and maturation. Previously, we have established that the promotion of larval growth and maturation by L. plantarum requires optimal expression of intestinal peptidases partially through PG-dependent Imd signalling. Imd-deficient individuals have impaired PG sensing; yet, in this context, L. plantarum still significantly promotes peptidase induction and larval growth. This observation indicates that L. plantarum must be detected by additional sensing.

**Fig. 5 | Sensing of multiple cell wall motifs is required for LpNC8-mediated larval growth promotion.** a. y,w and y,w,Dredd larval longitudinal length after inoculation with 10⁷ CFUs of LpNC8 (n=55), Δdltop (n=50) and PBS (n=45), for the germ-free condition. Larvae were collected five days after association and measured as described in the Methods. The bars in the graph represent means and s.d. NS represents the absence of a statistically significant difference; ****P<0.0001. Representative graph from one out of three independent experiments. b. Number of y,w and y,w,Dredd emerged pupae scored over time for eggs associated with strains LpNC8 and Δdltop and PBS for the germ-free condition. Forty germ-free eggs of each genotype were associated independently with 10⁷ CFUs of each one of the strains in ten biological replicates. The number of pupae was scored every 24h. A representative graph from one out of three independent experiments is shown. c. The day when 50% of the pupae emerge during a developmental experiment (D₅₀) for y,w and y,w,Dredd GF eggs associated with strains LpNC8 and Δdltop and PBS (for the GF condition). The central bars in the graph represent means. NS represents the absence of statistically significant difference; ***0.0001<P<0.001. d. Cell wall components of L. plantarum induce host intestinal transcriptional responses through a DAP-type PG-responsive NF-κB-dependent signalling pathway (PGRP-LE/Imd/Relish cascade) and yet unknown signalling cascade(s), triggered by direct sensing of cell walls bearing α-alanylated TA. Other bacterial signals beyond PG and α-alanylated TA contribute to ensure Drosophila optimal growth. For exact P values, see Supplementary Table 4.
mechanisms beyond PG recognition. In the current study, we show that the absence of pbbX2-dlxXABCD genes in \( Lp^{\Delta X} \) depletes d-alanine from TA and that cell walls deprived of d-alanylated TA lack the additional bacterial signal sensed by \( Drosophila \) enterocytes to trigger intestinal peptidase induction and systemic growth. Therefore, our results indicate that bacterial cell walls bearing d-alanylated TA are directly sensed by \( Drosophila \) enterocytes through a dual mechanism: PGRP-LE-mediated PG fragment recognition and Imd/Relish signalling; and direct sensing of d-alanylated TA and signalling by yet-to-be-discovered host mechanisms (Fig. 5d).

Previously, we established that promotion of intestinal peptidase expression and activity following \( L. \) \( \text{plantarum} \) association was necessary and sufficient to sustain host systemic growth; however, it is clear that the intestinal peptidase response does not account for the entire host growth promotion conferred by \( L. \) \( \text{plantarum} \). Our new study revealing a dual \( L. \) \( \text{plantarum} \)-sensing strategy by the host now paves the way to identify the full complement of the intestinal responses engaged following such sensing events and required besides intestinal peptidase induction to ensure optimal growth promotion. Our study also reveals that even if \( L. \) \( \text{plantarum} \)-sensing strategies are genetically altered (such as in the \( \text{Dredd} \) mutant associated with \( \Delta \text{dltop} \)), \( L. \) \( \text{plantarum} \) retains a residual but significant host growth-promotion capability, which remains elusive and will be the focus of future investigations.

Taken together, the results of our study unravel a central molecular dialogue engaged between chronically undernourished \( Drosophila \) and its commensal partner \( L. \) \( \text{plantarum} \), which supports the beneficial nature of their symbiosis. Given the recent demonstration of the conservation of the beneficial effects of \( L. \) \( \text{plantarum} \) on the linear growth of chronically undernourished gnotobiotic mouse models, our results pave the way to probing whether this molecular dialogue is conserved in mammals.

Methods

\( Drosophila \) diets, stocks and breeding. \( Drosophila \) stocks were cultured as described previously. Briefly, flies were kept at 25 °C with 12/12-hour dark/light cycles on a yeast/cornmeal medium containing 50 g l\(^{-1}\) of inactivated yeast. The poor-yeast diet was obtained by reducing the amount of inactivated yeast to 6 g l\(^{-1}\). Germ-free stocks were established as described previously. Axenicity was routinely tested by plating serial dilutions of animal lysates on nutrient agar plates.

\( Drosophila \) yw flies were used as the reference strain in this work. The following \( Drosophila \) line was also used: \( yw, \text{Dredd}^{\text{Δdlt}} \) (refs 11).

Bacterial strains and growth conditions. The strains used in this study are listed in Supplementary Table 1. \( E. \) \( \text{cherichica coli} \) strains were grown at 37 °C in LB medium with agitation. \( L. \) \( \text{plantarum} \) strains were grown in static conditions in MRS medium at 37 °C, unless stated otherwise. Erythromycin antibiotic was used at 5 \( \mu \)g ml\(^{-1}\) for \( L. \) \( \text{plantarum} \) and 150 \( \mu \)g ml\(^{-1}\) for \( E. \) \( \text{coli} \).

Random transposon mutagenesis of \( L. \) \( \text{plantarum} \). \( L. \) \( \text{plantarum} \) mutagenesis was performed using the \( P_{\text{pyr}}^{\text{Δ}} \)-\( \text{IpaeIS}_{\text{pum}} \) system as previously described\( ^{14,15} \). Briefly, electrocompotent \( L. \) \( \text{plantarum} \) cells were first transformed with \( pV1129 \), resulting in the NC\( pV1129 \) strain. Electrocompetent cells of the NC\( pV1129 \) strain were transformed with \( pV1110 \), plated on MRS plates supplemented with 5 \( \mu \)g ml\(^{-1}\) of erythromycin and incubated for 48 h at 42 °C to select for integrants. A total of 2,091 tn-insertion mutants were individually stored at -80 °C.

Library construction and deep sequencing. Genomic DNA was extracted from each transposon insertion mutant (UltraClean Microbial DNA isolation kit, MoBio). Twenty-two DNA pools were quantified using the Qubit system. The strains used in this study are listed in Supplementary Table 1.

Library screening for loss of the growth-promotion phenotype. The screen was performed in the poor-yeast diet. Each transposon insertion mutant (1 \( \times \) 10\(^6\) CFUs) was used to independently inoculate 20 \( \mu \)g germ-free eggs and incubated at 25 °C for 8 days. The number of pupae 8 days after egg laying was scored for each of the 2,091 tn-insertion mutants. Those values were converted to \( z \) scores (where \( \mu = 15.83 \equiv 2.091 \) and \( \sigma = 3.33 \)).

Sequence analysis and mapping of transposon insertion sites. \( pV1110 \) loci of insertion were confirmed for the eight loss-of-function mutants as described previously, with the following modifications. Genomic DNA was digested sequentially with Clal and BstXI restriction enzymes (NEB). Digested fragments were ligated using T4 DNA ligase (NEB) accordingly to the manufacturer’s instructions. Products of ligation were transformed into \( E. \) \( \text{coli} \) TGI thermo-competent cells, in which circularized fragments containing the transposon behave as plasmids. Plasmids were isolated and sequenced (Genewiz) with the primers OL221 and OL225 (Supplementary Table 3). Identification of transposon target sequences was performed with the BLAST software from the National Center for Biotechnology Information (NCBI).

Construction of \( ppbX2, \Delta \text{dltopXABCD} \) and \( \Delta \text{dltop} \) strains in \( L. \) \( \text{plantarum} \). Using \( pG \)-\( \text{host9} \), Independent markerless deletions on \( ppbX2 \) (\( \text{ApbX2} \), \( \text{dlitX} \) to \( \text{dlitXABCD} \)) and \( ppbX2 \) to \( \text{dlitXABCD} \) genes were constructed through homology-based recombination with double cross-over. Briefly, the 5′- and 3′-terminal regions of each region were PCR-amplified with Q5 High-Fidelity 2X Master Mix (NEB) and \( pV1129 \) to \( pV1129 \) (\( \text{ΔdltopXABCD} \)) were constructed through homology-based recombination with double cross-over. Briefly, the 5′- and 3′-terminal regions of each region were PCR-amplified with Q5 High-Fidelity 2X Master Mix (NEB) from \( L. \) \( \text{plantarum} \) NC8 chromosomal DNA. Primers contained overlapping regions with \( pG \)-\( \text{host9} \) (ref. 14) to allow for Gibson assembly. PCR amplifications were made using the following primers: OL118/OL119 and OL120/OL121 (\( ppbX2 \)), OL195/OL196 and OL197/OL198 (\( \text{dlitXABCD} \)), OL144/OL145 and OL146/OL147 (\( \text{dlitX} \)), listed in Supplementary Table 3. The resulting plasmids (Supplementary Table 1) obtained by Gibson assembly (NEB) were transformed into \( E. \) \( \text{coli} \) ECL1 competent cells and plated on MRS plates supplemented with 5 \( \mu \)g ml\(^{-1}\) of erythromycin. Overnight cultures grown under the same conditions were diluted and shifted to the non-permissive temperature (42 °C) in the presence of 5 \( \mu \)g ml\(^{-1}\) of erythromycin to select single-cross-over integrants. Plasmid excision by a second recombination event was promoted by growing integrants at the permissive temperature without erythromycin. Deletions were confirmed by PCR followed by sequencing with primers OL126/OL127 (\( ppbX2 \)), OL126/OL128 (\( \text{dlitXABCD} \)) and OL148/OL149 (\( \text{dlitX} \)).
Quantification of d-alanine from TAs by high-performance liquid chromatography (HPLC). d-Alanine esterified to TAs was detected and quantified as described previously. Briefly, d-alanine was released from whole heat-inactivated bacteria by mild alkaline hydrolysis with 0.1% NaOH for 1 h at 37°C. After neutralization, the extract was incubated with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-l-alanine amide; Sigma). This reagent reacts with the optical isomers of amino acids to form diastereomeric N-aryl derivatives, which can be separated by HPLC. Separation of the amino-acid derivatives was performed on a C4 reversed-phase column (Zorbax Eclipse Plus C18 RRHD 2.1 × 30mm, 1.8 μm Agilent) with an Agilent UHPLC 1290 system with a linear elution gradient of acetonitrile in 20 mM sodium acetate buffer (pH 4) as described previously. The eluted compounds were detected by ultraviolet absorbance at 340 nm.

Quantification was achieved by comparison with d-alanine standards in the range of 100 to 1,500 pmol. Mean values were obtained from five independent cultures with two injections for each.

Purification of L. plantarum cell wall. L. plantarum cells were grown in MRS medium and harvested at mid-exponential phase. After being washed with MilliQ water, the cells were boiled for 10 min and centrifuged at 5,000g for 4°C. CFUs (3.75×10^9) were resuspended in 1 ml of SDS 5% in 50 mM of MES (Sigma-M8250) at pH 6.0, pre-heated at 60°C and boiled for 25 min. After centrifugation at 20,000g, the pellets were resuspended in 1 ml of SDS 5% in 50 mM of MES pH 5.5, pre-heated at 60°C and boiled for 15 min and then mixed with MilliQ water to remove SDS traces. Next, the pellets were sequentially enzymatically treated with 2mg/ml of Pronase (Roche 165921) in 50 mM of MES (Sigma-M8250) at pH 5.5, pre-heated at 60°C, 200μg/ml of trypsin (Sigma-T-0030) in 50 mM of MES (Sigma-M8250) for 2 h at 37°C with shaking; DNase (Sigma-D-4527) and RNase (Sigma-R-5503) (50 μg/ml) in 50 mM of MES (Sigma-M8250) at pH 6.0 for 1 h at 37°C. After boiling for 15 min with SDS 2% in 50 mM of MES (Sigma-M8250) at pH 5.5 and centrifugation at 20,000g for 10 min, the pellets were washed with MilliQ water to remove SDS traces. Purified cell walls (containing PG, WTA and polysaccharides) were finally lyophilized until use and for mass determination. We have confirmed that d-alanine esterification was kept on WTA in the purified cell walls of WT LpCW, whereas it was absent in the Δdltop mutant cell walls, using the protocol described above.

Purified cell wall quantification. Purified cell walls were quantified by measuring the muramic acid content in peptidoglycan after acid hydrolysis. Purified cell walls (200μg) were suspended in 1 M HCl in vacuum conditions. The samples were then dried under vacuum conditions and resuspended in 100 μl MilliQ H2O. Muramic acid was quantified by high-performance anion exchange chromatography coupled with pulse amperometric detection (HPAEC-PAD) as described previously, with an ICS5000 system (Thermo Fisher). Samples (20μl) were injected on a Dionex CarboPac PA-1 anion exchange column (4×250 mm) (Thermo Fisher) with a guard column and eluted at a flow of 1 ml min⁻¹ and a temperature of 30°C. Three eluents were used: MilliQ H2O (eluant A), 150 mM NaOH (eluant B) and 150 mM sodium acetate in 100 mM NaOH (eluant C). The separation of muramic acid from other cell wall sugars and amino acids was achieved by a first isocratic step of 20% eluent B for 6 min followed by a linear gradient (0–100%) of eluant C over 40 min, and finally with 100 % eluent C for 5 min. PAD detection operated with a gold working electrode and a Ag/AgCl combination reference electrode (Thermo Fisher). Quantification was carried out with a standard curve of pure muramic acid (Sigma-Aldrich) (50 to 1,000 pmol). The amount of PG in purified cell walls from LpCW and Δdltop cells was determined as 151 nmol mg⁻¹ and 162 nmol mg⁻¹, respectively.

Larval size measurements. Axenic adults were placed in breeding cages overnight to lay eggs on sterile poor-yeast diet. Fresh axenic embryos were collected the next morning and seeded in pools of 40 on 35 mm Petri dishes containing fly food. CFUs (1×10⁹, unless specified otherwise) or PBS was then inoculated homogeneously on the substrate and the eggs. The Petri dishes were incubated at 25°C until collection of larvae. Drosophila larvae, seven days after inoculation (unless specified otherwise), were randomly collected and processed as described previously. Larval longitudinal length of individual larvae was quantified using ImageJ software. For the cell wall experiments, supplemented, 170 nmol of purified cell wall (resuspended in PBS; corresponding to the amount of cell wall extracted from 10⁶ CFUs), was added to the fly food every day until day 6.

Developmental timing determination. Axenic adults were placed in breeding cages overnight to lay eggs on sterile poor-yeast diet. Fresh axenic embryos were collected the next morning and seeded by pooling 40 in tubes containing fly food. A total of 1×10⁶ CFUs of each strain (unless specified otherwise) or PBS was then inoculated homogeneously on the substrate and the eggs and incubated at 25°C. The emergence of pupae was scored every day until all pupae had emerged. D₅₀ was determined using D50App (http://www.paulinejoncour.shinyapps.io/D50App), which is a Shiny app that calculates, for the pupae emerged during a developmental experiment, the day when 50% of the pupae emerged. It takes as input a table with experiment, the day when 50% of the pupae emerged. The samples were then contrasted with oolong tea extract 0.5% in cotate buffer, postfixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate, gradually dehydrated in an ethanol series (30% to 100%) and substituted gradually in a mix of ethanol–Epon–epoxy and embedded in Epon (Delta Microscope). Thin sections (70 nm) were collected onto 200 mesh cooper grids, and counterstained with lead citrate. The grids were examined with a Hitachi HT7700 electron microscope operated at 80 kV (Elexience), and images were acquired with a CCD camera (AMT). Transmission electron microscopy analyses were performed at the Microscopy and Imaging Platform MIMA2 (INRA, Jouy-en-Josas, France). The cell width at half the cell length (mid-width) was determined by ImageJ using the Microble plug-in.

Transmission electron microscopy. Pellets of bacteria were fixed with 2% glutaraldehyde in 0.1 M Na cacodylate buffer at pH 7.2 for 1 h at room temperature. Samples were then contrasted with oolong tea extract 0.5% in cotate buffer, postfixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate, gradually dehydrated in an ethanol series (30% to 100%) and substituted gradually in a mix of ethanol–Epon–epoxy and embedded in Epon (Delta Microscope). Thin sections (70 nm) were collected onto 200 mesh cooper grids, and counterstained with lead citrate. The grids were examined with a Hitachi HT7700 electron microscope operated at 80 kV (Elexience), and images were acquired with a CCD camera (AMT). Transmission electron microscopy analyses were performed at the Microscopy and Imaging Platform MIMA2 (INRA, Jouy-en-Josas, France). The cell width at half the cell length (mid-width) was determined by ImageJ using the Microble plug-in.

Intestinal peptidase activity. Five biological replicates of five midguts per condition were dissected in 100 μl of PBS and homogenized using a dounce homogenizer. The homogenate was then centrifuged at 10,000g for 30 min at 4°C. The 10,000g supernatant represents the crude luminal and cytosolic fraction. Protein determinations were made using the BCA method (Pierce), with bovine serum albumin as a standard. Chymotrypsin activity of the peptidase was assayed using the fluorogenic peptide Ala-Ala-Phe-7-aminomethylcoumarin (25 μM). The assay was carried out using 10 μg of cytosolic protein in a total volume of 200 μl. The assay buffer was composed of 25 mM Tris-HCl at pH 7.5 and contained the appropriate peptide substrate. Enzymatic kinetics was conducted in a temperature-controlled microplate fluorimeter reader (Fluostar Galaxy, BMG). Activity was measured by the release of t-aminomethyl-coumarin fluorescence over time (350/440 nm excitation/emission wavelengths). Activity is expressed as the quantity of fluorescence emission per minute per microgram of protein.

RNA extraction and RT-qPCR analysis. Axenic y,w,y,w,Dред eggs were inoculated with 1×10⁶ CFUs of LpCW and Δdltop strains independently or kept axenic. Larvae were size-matched for the three conditions and harvested at mid-3 larval staged kept axenic until mid-3 stage and were inoculated with 170 nmol of purified cell wall from LpCW and Δdltop for 6 and 24 h. RNA extraction of five replicas of six dissected guts for each condition was performed as described previously. Quantitative PCR with reverse transcription.
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**Data availability.** The data that support the findings of this study are available from the corresponding author upon request.

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**Author contributions**

F.L. supervised the work. R.C.M. and F.L. designed the experiments. R.C.M., M.S., A.-L.B., D.M. and H.G. performed the experiments. B.G. and S.H. designed and performed high-throughput insertion tracking by deep sequencing. M.E.M. and P.J. performed the insertion site bioinformatics analysis. P.C. performed d-alanine and PG quantifications. A.-L.B. developed the protocol for proteolytic activity determination. R.C.M., A.-L.B., P.C., M.-P.C.-C., M.S. and F.L. analysed the results. R.C.M. and F.L. wrote the manuscript.

**Competing interests**

The authors declare no competing financial interests.

**Additional information**

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