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Probiotic *Lactobacillus rhamnosus* Reduces Organophosphate Pesticide Absorption and Toxicity to *Drosophila melanogaster*

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ABSTRACT

Organophosphate pesticides used in agriculture can pose health risks to humans and wildlife. We hypothesized that dietary supplementation with *Lactobacillus*, a genus of commensal bacteria, would reduce absorption and toxicity of consumed organophosphate pesticides (parathion and chlorpyrifos [CP]). Several *Lactobacillus* species were screened for toleration of 100 ppm of CP or parathion in MRS broth based on 24-h growth curves. Certain *Lactobacillus* strains were unable to reach stationary-phase culture maxima and displayed an abnormal culture morphology in response to pesticide. Further characterization of commonly used, pesticide-tolerant and pesticide-susceptible, probiotic *Lactobacillus rhamnosus* strain GG (LGG) and *L. rhamnosus* strain GR-1 (LGR-1), respectively, revealed that both strains could significantly sequester organophosphate pesticides from solution after 24-h coincubations. This effect was independent of metabolic activity, as *L. rhamnosus* GG did not hydrolyze CP and no difference in organophosphate sequestration was observed between live and heat-killed strains. Furthermore, LGR-1 and LGG reduced the absorption of 100 μM parathion or CP in a Caco-2 Transwell model of the small intestine epithelium. To determine the effect of sequestration on acute toxicity, newly eclosed *Drosophila melanogaster* flies were exposed to food containing 10 μM CP with or without supplementation with live LGG. Supplementation with LGG simultaneously, but not with administration of CP 3 days prior (prophylactically), mitigated CP-induced mortality. In summary, the results suggest that *L. rhamnosus* may be useful for reducing toxic organophosphate pesticide exposure via passive binding. These findings could be transferable to clinical and livestock applications due to affordability and practical ability to supplement products with food-grade bacteria.

IMPORTANCE

The consequences of environmental pesticide pollution due to widespread usage in agriculture and soil leaching are becoming a major societal concern. Although the long-term effects of low-dose pesticide exposure for humans and wildlife remain largely unknown, logic suggests that these chemicals are not aligned with ecosystem health. This observation is most strongly supported by the agricultural losses associated with honeybee population declines, known as colony collapse disorder, in which pesticide usage is a likely trigger. Lactobacilli are bacteria used as beneficial microorganisms in fermented foods and have shown potentials to sequester and degrade environmental toxins. This study demonstrated that commonly used probiotic strains of lactobacilli could sequester, but not metabolize, organophosphate pesticides (parathion and chlorpyrifos). This *Lactobacillus*-mediated sequestration was associated with decreased intestinal absorption and insect toxicity in appropriate models. These findings hold promise for supplementing human, livestock, or apiary foods with probiotic microorganisms to reduce organophosphate pesticide exposure.

Organophosphate pesticides are a class of insecticide under scrutiny for being linked to toxic effects in both humans and wildlife (1). However, these compounds are still commonly used in agriculture and pest control programs. Organophosphate pesticides include parathion, malathion, methyl parathion, chloropyrifos (CP), diazinon, dichlorvos, phosmet, fenitrothion, tetrachlorvinphos, azamethiphos, and azinphos-methyl. Organophosphate pesticides irreversibly inhibit acetylcholinesterase to induce excessive cholinergic stimulation (2, 3). The potential off-target health consequences of organophosphate pesticide exposure for humans include neonatal developmental abnormalities (4, 5), endocrine disruption (6), neurodegeneration, cancer (7, 8), metabolic disruption (9), heart disease (10), chronic kidney disease, and other less common pathologies. In addition, organophosphate pesticides are reported to have negative impacts on honeybee colonies, which are critical pollinators for numerous agricultural products (11–13). Although the evidence is mixed and often correlative, the original use of these organophosphates as nerve agents strongly suggests that these pesticides are not aligned with human or wildlife health.

Despite negative consequences, the affordability and need to prevent crop losses associated with insect infestations suggest that

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organophosphate pesticide use will continue in the near future. One long-considered counter to these effects is the use of microbes to detoxify organophosphate pesticide-contaminated environments (14–20). Classical bioremediation efforts have identified numerous strains of soil bacteria that contain genetically diverse phosphotriesterases capable of organophosphate degradation (15). Moreover, the symbiotic relationship between *Burkholderia* strains and the bean bug, *Riportus pedestris*, has been shown to confer upon these insects resistance to organophosphate insecticides (21). Taken together, these observations suggest that microorganisms could be used to reduce the toxic effects of organophosphate insecticides *in vivo*.

There have been developments within bioremediation research to investigate the potential of transitory food-grade bacteria to prophylactically prevent the absorption of environmental toxins, such as pesticides (22), heavy metals (23), and aflatoxin (24, 25). Many lactobacilli are commonly used in fermented foods, such as yogurt, cheese, sauerkraut, pickles, beer, wine, cider, kimchi, cocoa, and kefir (26). Lactobacilli have also been shown to be natural and beneficial gastrointestinal microbiota members in humans, livestock, honeybees (27), and fish (28). Notably, lactobacilli have been shown to reduce organophosphate pesticide contamination of dairy products (29, 30). The mechanism of action used by lactobacilli against organophosphate pesticides remains unclear and is commonly attributed to phosphatase capabilities. However, one kimchi isolate, *Lactobacillus brevis* WCP902, was shown to contain the *opdB* gene, which conferred the active ability to degrade CP (31). Similarly, the common probiotic *Lactobacillus rhamnosus* strain GG (LGG) has a predicted hydrolase/phosphotriesterase (LGG_RS02045) with sequence similarity to the experimentally validated parathion hydrolase (*opd*) found in *Brevundimonas diminuta*.

The aim of this study was to better characterize the *in vivo* bioremediation potential of probiotic food-grade bacteria that interact with organophosphate pesticides. We hypothesized that the direct binding or metabolism of organophosphate pesticides by *L. rhamnosus* strains would reduce toxin absorption and downstream organism toxicity.

MATERIALS AND METHODS

Chemicals. CP (catalog number 45395) and parathion (catalog number 45607) were obtained from Sigma-Aldrich. Stock solutions were prepared at 100 mg/ml or 10 mg/ml in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored frozen at −20°C until usage.

Bacterial strains and culture. *Lactobacillus* species and strains used were *L. rhamnosus* GG, *L. rhamnosus* GR-1, *L. casei* ATCC 393, *L. delbrueckii* DSM 20074, *L. plantarum* ATCC 14917, *L. crispatus* ATCC 33820, *L. fermentum* ATCC 11739, *L. johnsonii* DSM 20053, *L. reuteri* ATCC 2773, and *L. rhamnosus* ATCC 7469.

A pET20b vector (EMD Millipore) containing the organophosphate insecticide-degrading *PTE* (phosphotriesterase) gene inserted between NdeI and EcoRI sites (20) was obtained as a gift from Frank M. Raushel (Texas A&M University, USA). Three hundred nanograms of pET20b/*PTE* vector was transformed into 50 μl of chilled (on ice for 15 min) chemically competent *Escherichia coli* BL21(DE3) cells by 2 min of heat shock at 42°C followed by incubation on ice for 2 min. The transformation mixture was serially diluted and plated for positive selection on Luria-Bertani agar (LB, catalog number DF0446173; BD Difco) plates containing 300 μg ampicillin/ml. Plasmids were isolated from transformants grown at 37°C aerobically overnight using the PureLink Quick plasmid miniprep kit according to the manufacturer's instructions (catalog number K210010; Invitrogen). Positive transformants were confirmed by sequencing the plasmids with the T7 promoter (TAATACGACTCACTATAGGG) and T7 terminator (GCTAGTTATTGCTCAGCGG) primers with the Applied Biosystems 3730 Analyzer platform at the London Regional Genomics Centre (Robart's Research Institute, London, Canada).

E. coli BL21(DE3)(pET20b/*PTE*) cells were inoculated from LB agar plates into LB broth, both supplemented with 300 μg ampicillin/ml of culture medium and incubated overnight (18 h) at 37°C aerobically. Lactobacilli were inoculated from Lactobacilli MRS agar (de Man, Rogosa, and Sharpe agar [catalog number 288130]; BD Difco) plates into MRS broth. Inoculated MRS broth cultures were subcultured and incubated overnight (18 h) at 37°C anaerobically and statically for experimental procedures. Bacteria were heat killed by incubation at 56°C for 90 min when necessary. Bacterial killing was confirmed by spread plating 100 μl of bacterial culture on MRS agar plates and verifying the absence of colony growth after 3 days (37°C anaerobically).

Pesticide tolerance assay. Overnight broth cultures (stationary phase, 10^9 CFU/ml) were subcultured (1:100 dilution) into 96-well plates (catalog number 351177; Falcon) containing MRS broth with or without the addition of 100 ppm CP or parathion or vehicle (DMSO). Plates were sealed with optically clear multiwell plate-sealing films and incubated at 37°C and read every 30 min for 24 h at a wavelength of 600 nm by using a Labsystems Multiskan Ascent microplate reader.

Pesticide hydrolysis assay. Semiqualitative assessment of bacterial CP hydrolysis was determined based on a modified protocol previously described (21). Briefly, 1 μl of overnight broth culture (10^6 CFU) of LGG or *E. coli* BL21(DE3)(pET20b/*PTE*) (positive control) was spot plated onto brain heart infusion (catalog number B11059; DB Difco) agar plates containing 1,000 ppm (1 mg/ml) of emulsified CP. After 48 h of anaerobic or aerobic incubation at 37°C for LGG and *E. coli* BL21(DE3) pET20b/*PTE*, respectively, the radius of halo formation (zone of clearing) was determined.

Pesticide binding and metabolism assay. Overnight bacterial cultures were pelleted at 5,000 × *g*, washed, and resuspended in 50 mM HEPES (pH 6.8; 10^9 CFU/ml). Bacteria-buffer or buffer-alone solutions were incubated with 100 ppm of parathion or CP protected from light at 37°C for 24 h with gentle shaking at 200 rpm. Cultures were pelleted and supernatant was collected. Pellets were washed in 50 mM HEPES, resuspended in methanol, and sonicated for 15 min. Supernatants were again collected for assessment of organophosphate levels in pellets.

Pesticide levels were determined by high-performance liquid chromatography (HPLC) using a Poroshell 120 column (100 by 4.6 mm, 2.7 μm; Agilent, Mississauga, Ontario, Canada) and a UV detector, based on a modified protocol (32). CP and parathion were detected at 288- and 275-nm wavelengths, respectively. The solvent flow rate was 1 ml/min for solvent A (H_2O plus 0.1% trifluoroacetic acid) and solvent B (acetonitrile plus 0.1% trifluoroacetic acid). A solvent gradient was set to 25, 25, 100, 100, 25, and 25% B at 0, 2, 9, 11, 11.5, and 13 min, respectively. Sample peak area units were compared to a linear relationship of appropriate standards of known concentrations. The limits of detection for parathion and CP were 0.001 ppm and 0.02 ppm, respectively. The percentage of pesticide remaining was calculated as the concentration (parts per million) of biological replicate divided by the concentration (parts per million) of the mean pesticide-only control replicates × 100.

Caco-2 cell culture and Transwell experimentation. Caco-2 cells were obtained as a gift from Brad Urquhart (Western University, London, Canada). Caco-2 cells were routinely maintained at 37°C under atmospheric conditions with 5% CO_2. Cells were cultured in Dulbecco's modified Eagle medium (catalog number 11960044; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1 mM sodium pyruvate (catalog number 11360070; Gibco), 1% MEM nonessential amino acids (catalog number 11140050; Gibco), 4 mM L-glutamine (catalog number 25030081; Gibco), and 100 U/ml penicillin-streptomycin (catalog number 15140122; Gibco). Cells were used experimentally between passages 35 and 45.

Cells were seeded onto 12-mm Transwell plates (12-well plates with a 0.4-μm-pore-size polyester membrane insert, catalog number 3460; Corning) at a concentration of 1.5×10^5 cells/insert. Cells were differentiated by culture for 21 days as previously described (33). Prior to absorption experimentation, apical and basolateral compartments were washed 3 times and resuspended in Hanks' balanced salt solution (catalog number 14025092; Gibco). Cells were treated apically with 100 μM parathion or CP in the presence or absence of 10^9 CFU/ml of LGG or LGR-1. Basolateral sampling (100 μl) with replacement was performed at 30 min, 1 h, and 2 h. Following absorption experimentation, Caco-2 monolayer integrity was confirmed in a Lucifer yellow (CH lithium salt, catalog number 80015; Biotium) rejection assay according to the manufacturer's instructions.

***Drosophila melanogaster* husbandry.** Wild-type Canton-S (stock number 1) stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University. *Drosophila* flies were maintained in medium containing 1.5% (wt/vol) agar, 1.73% (wt/vol) yeast, 7.3% (wt/vol) cornmeal, 7.6% (vol/vol) corn syrup, and 0.58% (vol/vol) acid mix at 22°C with 12-h light/dark cycles. For experimental procedures, media were supplemented with or without CP at concentrations of 1, 10, or 100 μM prior to solidification. Food was stored at 4°C and used within a week. Experiments with adult *Drosophila* used newly eclosed flies. All experiments were performed in polypropylene Drosophila vials (GEN32-121 and GEN49-102; Diamed Lab Supplies Inc.) containing 10 ml of food with 15 to 25 flies/tube.

Adult *Drosophila* survival assays. Fifteen to 25 newly eclosed *Drosophila* flies were anesthetized with CO_2 and randomly transferred (mixed sex) into standard vials at mid-light cycle (9 a.m.) for each replicate. *Drosophila* flies were confirmed to be alive following anesthetization and subsequently monitored daily (9 a.m.) for survival. Medium was supplemented with a single dose of 100 μl (10^9 CFU) of washed and concentrated LGG or phosphate-buffered saline vehicle when experimentally appropriate and allowed to air dry before use.

***Drosophila* negative geotaxis assay.** Negative geotaxis assays were performed as previously described (34). The mean distances climbed (after 3 s) of 5 replicates from 3 independent experiments were determined.

***Drosophila* microbiota analysis.** Whole *Drosophila* flies were surface sterilized with 70% ethanol and homogenized with a handheld motorized pestle in 0.01 M phosphate-buffered saline. Serially diluted fly homogenates were spot plated onto MRS agar plates. Plates were incubated anaerobically at 37°C, and CFU were enumerated after 48 h of incubation.

Statistical analyses. All statistical analyses were performed using GraphPad Prism 6 with one-way analysis of variance tests with Tukey's multiple comparisons test or the Kruskal-Wallis test with Dunn's multiple-comparisons test. Alternatively, Mantel-Cox and Gehan-Breslow-Wilcoxon tests (in which early time points are weighted more strongly) were used for *Drosophila* survival analyses.

RESULTS

Lactobacilli vary in their ability to tolerate high levels of organophosphate pesticides. In an effort to determine a strain of *Lactobacillus* able to tolerate high levels of organophosphate pesticides, lactobacilli were screened for the ability to tolerate 100 ppm of parathion or CP via growth curves. In general, lactobacillus growth was largely unaltered by the presence of parathion or CP relative to the vehicle control. Based on the solubility maxima of parathion and CP at 37°C, pesticide concentrations greater than 100 ppm were not evaluated. However, LGR-1, *L. casei* ATCC 393, and *L. delbrueckii* DSM 20074 demonstrated significantly reduced growth ($P < 0.05$) in the presence of parathion or CP (Fig. 1A). Organophosphate pesticide treatment significantly reduced the stationary-phase growth maxima of these strains of lactobacilli compared to the vehicle control treatment (Fig. 1B). The organophosphate-induced growth deficiency was associated with abnormal shiny and mucoid culture morphologies for *L. casei* ATCC 393 and *L. delbrueckii* DSM 20074 (Fig. 1C). However, quantification of biofilm formation in a microtiter dish assay did not demonstrate any observable differences in biofilm formation for LGG, LGR-1, *L. casei* ATCC 393, or *L. delbrueckii* DSM 20074 following a 24-h exposure to 100 ppm of parathion or CP (see Fig. S1 in the supplemental material).

Hydrolase (WP_014569076.1) in *L. rhamnosus* GG was computationally identified as a potential organophosphate hydrolase. The National Center for Biotechnology Information's align sequences protein Basic Local Alignment Search Tool (standard settings) was used to compare the query WP_014569076.1 (hydrolase corresponding to the gene LGG_RS02045 or *php*) to the experimentally confirmed parathion hydrolase present in *Brevundimonas diminuta* (UniProtKB P0A434). A significant alignment was observed ($E = 10^{-26}$) with the standard compositional matrix adjustment method with the following characteristics: score = 94.4 bits, identities = 73/268, positives = 134/268 (50%), and gaps = 30/268 (11%). The standard settings for T-Coffee (version 11.00.d625267; 2016-01-11, 15:25:41, revision d625267, build 507) (35) were used to generate a multiple-sequence alignment between the hydrolase WP_014569076.1 from LGG and the experimentally confirmed parathion hydrolases from *B. diminuta* and *Sphingobium fuliginis* ATCC 27551 (UniProtKB P0A433) (Fig. 2). In addition, protein modeling with the Protein Homology/analogY Recognition Engine software version 2.0 (36) best matched the LGG hydrolase WP_014569076.1 as a likely member of the metallo-dependent hydrolases and phosphotriesterase-like protein superfamily and family, respectively, with 100% confidence and 52% identity.

***L. rhamnosus* strains GG and GR-1 can bind, but not metabolize, organophosphate pesticides.** The functional potential of the hydrolase WP_014569076.1 from LGG was assessed in the semiquantitative pesticide hydrolysis assay with CP. Compared to the positive control, *E. coli* BL21(DE3)(pET20b/*PTE*), LGG did not demonstrate any CP hydrolysis under the aforementioned culture conditions, as evidenced by the lack of halo formation (Fig. 3). These results suggested that the hydrolase WP_014569076.1 likely does not function as an organophosphate pesticide hydrolase.

The commonly used commercial probiotics, LGG and LGR-1, were also characterized for organophosphate pesticide binding or metabolism, since they displayed opposite phenotypes of unimpaired and impaired growth in organophosphate-containing media, respectively. Both LGG and LGR-1 significantly sequestered parathion and CP from solution ($P < 0.05$); however, this effect was much more pronounced with CP (Fig. 4A). Interestingly, organophosphate sequestration was observed with LGG and LGR-1 even after heat killing, and the extent of binding was similar to that for live bacteria (Fig. 4A). This finding further supported the idea that the predicted LGG organophosphate hydrolase gene, WP_014569076.1, is not involved in organophosphate pesticide metabolism. The binding of organophosphate pesticides by LGG and LGR-1 was further supported by the confirmation of parathion and CP in the bacterial pellets following experimentation (Fig. 4B). The amount of CP observed in LGG pellets was considerably higher than that in LGR-1 pellets. A discrepancy between the amount of CP sequestered by LGR-1 and that found in LGR-1 pellets was likely due to weak LGR-1–CP binding interactions that were perturbed by washing the pellets

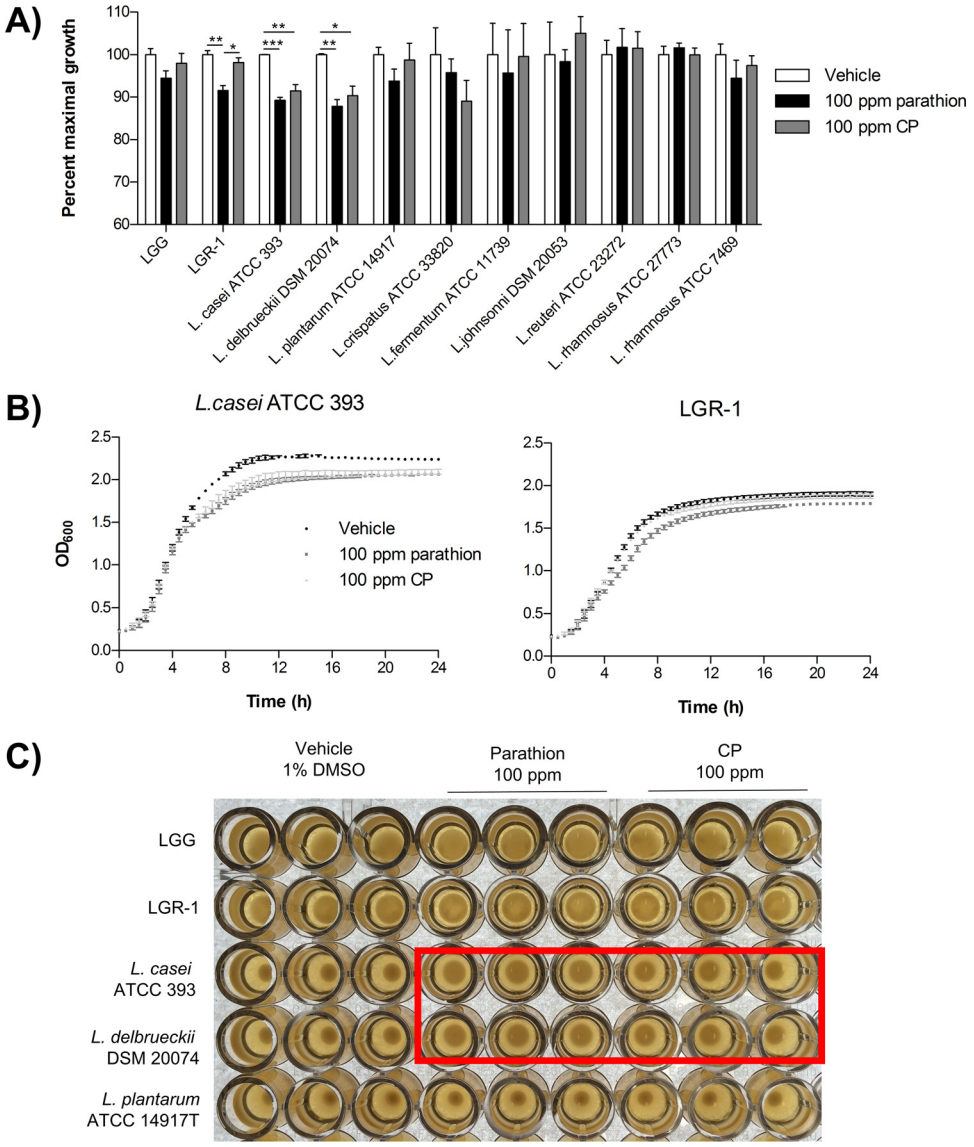


FIG 1 Lactobacilli vary in their ability to tolerate high levels of organophosphate pesticides. (A) Percent maximal growth was calculated from 24-h growth curve data (based on the optical densities at 600 nm) using the area under the growth curve of pesticide-treated bacteria relative to that of vehicle treatment of each bacterial strain. (B) Representative growth curves of *Lactobacillus casei* ATCC 393 and *Lactobacillus delbrueckii* DSM 20074. (C) Representative image of lactobacillus cultures following 24 h of treatment with 100 ppm parathion or CP or vehicle. Red box, *L. casei* ATCC 393 and *L. delbrueckii* DSM 20074 cultures with differential morphologies following organophosphate pesticide treatment, compared to the vehicle cultures. Data are mean (± standard error of the mean) results from 3 independent experiments with triplicate technical replicates.

prior to lysis. This preferential interaction of LGG, rather than LGR-1, with CP was not observed with parathion. Furthermore, disrupting bacterial membranes and surface proteins by using methanol as a solvent (37) for binding experiments prevented any notable observations of bacterium-organophosphate binding (Fig. 4C).

***L. rhamnosus* GG and GR-1 reduced the absorption of organophosphate pesticides in a Caco-2 Transwell model.** The ability of LGG- and LGR-1-mediated organophosphate pesticide sequestration was tested for the potential to reduce intestinal absorption of these compounds via a Caco-2 Transwell model of the small intestine. LGG and LGR-1 moderately reduced the Caco-2 apical-basolateral translocation of both parathion and CP compared to unsupplemented controls (Fig. 5). Similar to earlier binding experiments, reduction of organophosphate pesticide absorption was more pronounced with LGG than with LGR-1, and lactobacillus binding was more prominent with CP than with parathion. Basolateral levels of parathion increased kinetically (30 min > 60 min > 120 min) post-apical exposure for cells exposed to CP with or without supplementation of LGG or LGR-1. However, there was an insignificant trend (other than that for CP compared to CP-LGG at 1 h) of decreased basolateral parathion levels in LGG- or LGR-1-supplemented cells at all time points. Most notably, at 1 h post-apical exposure, CP apical-basolateral absorption was undetectable in CP-LGG and CP-LGR-1 simultaneously treated cells compared to cells treated with CP alone. However, by

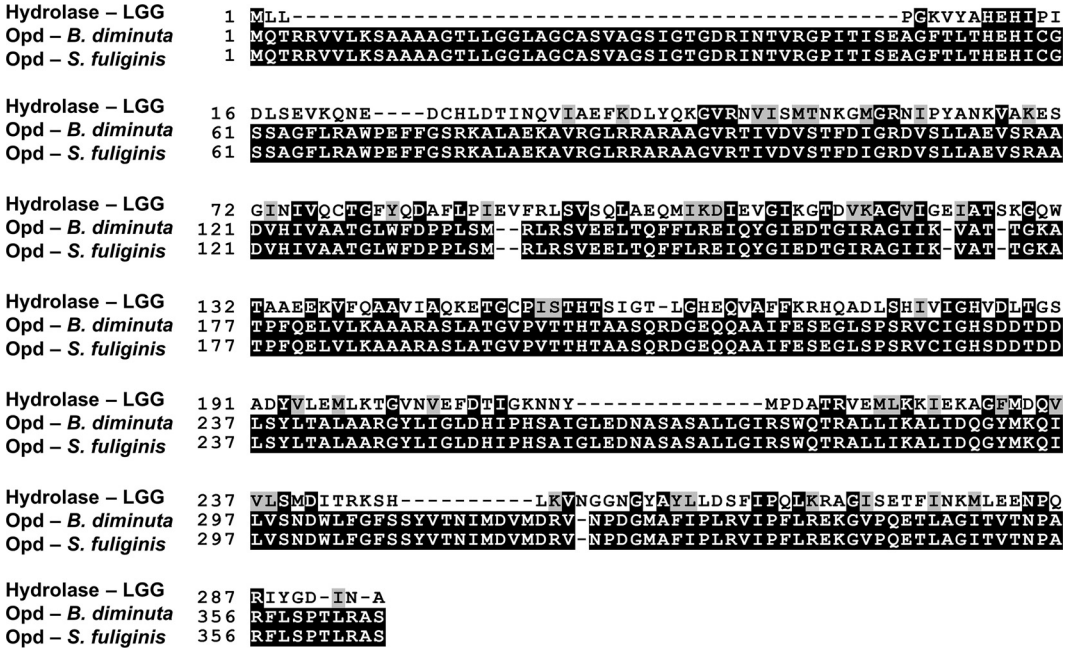


FIG 2 Multiple-sequence alignment of the LGG hydrolase, WP_014569076.1, and experimentally confirmed parathion hydrolases (Opd) from *B. diminuta* and *S. fuliginis* ATCC 27551.

2 h post-apical exposure, basolateral CP levels in CP-LGG– and CP–LGR-1–treated cells increased notably but remained significantly lower ($P < 0.05$) than that for cells treated with CP alone. These findings support earlier observations of potentially biologically relevant interactions of probiotic lactobacilli with consumed organophosphate pesticides that seem to vary in effect based on subtle differences in molecular structure.

***L. rhamnosus* GG supplementation reduced the toxicity of chlorpyrifos to *Drosophila*.** Since LGG displayed both the greatest sequestration and reduction of *in vitro* intestinal absorption of CP, the ability of oral LGG supplementation to mitigate mortality of *Drosophila melanogaster* exposed to food containing a lethal amount of CP was tested. Adult *Drosophila* flies were exposed to CP-containing food at various concentrations to determine an optimal CP dosage for intervention testing. *Drosophila* flies were sensitive to CP-induced mortality in a dose-dependent manner (Fig. 6A). Median survival of *Drosophila* was determined to be 1 day for 100 μM CP, 3 days for 10 μM CP, and undeterminable for 1 μM CP or vehicle. Based on these dose-mortality experiments, the 10 μM CP concentration was chosen to investigate the ability of LGG to mitigate acute CP-induced mortality. *Drosophila* flies that were simultaneously exposed to LGG and 10 μM CP had a median survival comparable to that of *Drosophila* flies that received just 10 μM CP (for both, median survival was 3 days) (Fig. 6B). However, the *Drosophila* flies simultaneously supplemented with both LGG and 10 μM CP displayed significantly prolonged overall survival (log rank test [Mantel-Cox], chi-square = 15.45, degrees of freedom [df] = 1; $P < 0.0001$) and fewer early deaths (Gehan-Breslow-Wilcoxon test, chi-square = 7.361, df = 1; $P < 0.01$) than those treated with 10 μM CP alone (Fig. 6B). Notably,

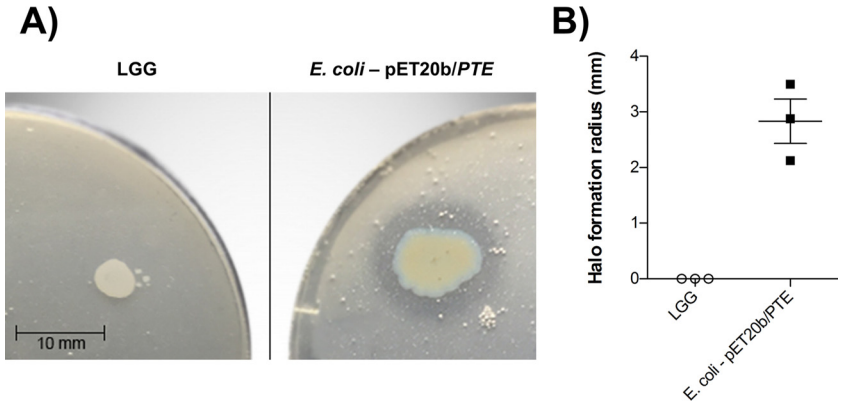


FIG 3 The LGG hydrolase WP_014569076.1 does not hydrolyze organophosphate pesticides. (A) Representative image of results for the semiquantitative pesticide hydrolysis assay following 48-h incubations. (B) The radius of halo formation (pesticide hydrolysis) was quantified following 48-h incubations. Data are presented as means ± standard errors of the means from results of 3 independent experiments containing 3 or 4 technical replicates.

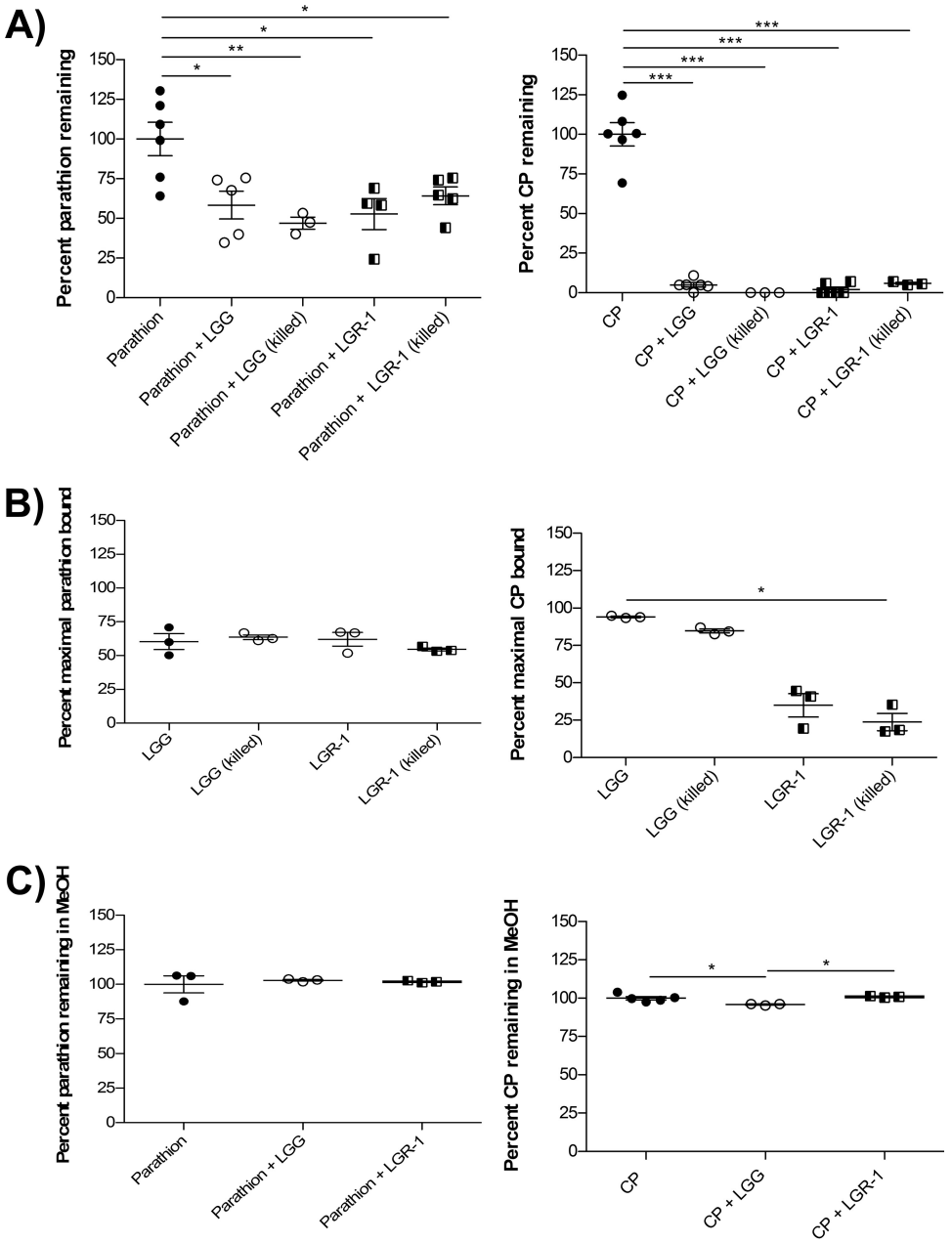


FIG 4 LGG and LGR-1 can bind, but not metabolize, organophosphate pesticides. (A) Percentages of parathion and CP metabolized were determined in stationary-phase live and heat-killed LGG and LGR-1 cultures, relative to results in pesticide-only controls following 24-h coincubations in 50 mM HEPES. (B) Percent maximal parathion and CP bound relative to 100 ppm input was determined in bacterial pellets following 24-h pesticide coincubations in 50 mM HEPES. (C) Percentages of parathion and CP metabolized were determined in stationary-phase chemically killed LGG and LGR-1 cultures, relative to pesticide-only controls, following 24-h coincubations in methanol. Data are means ± standard deviations of results from at least 3 independent experiments *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

at the experimental endpoint (day 12), 9.901% of *Drosophila* flies that were supplemented with LGG were still alive; alternatively, 0% of *Drosophila* flies exposed to 10 μM CP alone survived to 10 days postexposure. These observations suggest that prophylactic priming and continual supplementation of *Drosophila* with LGG may maximize *Drosophila* survival by preventing early deaths associated with CP exposure.

Further experimentation was conducted in an attempt to elucidate if the prosurvival effects observed in CP-exposed *Drosophila* flies supplemented with LGG were due to direct LGG-CP interactions or to LGG modification of host responses to pesticide toxicity. To test this, *Drosophila* flies were pretreated with vehicle or LGG for 3 days prior to being transferred to medium containing 10 μM CP. No overall survival benefit (log rank test [Mantel-Cox], chi-square = 0.1419, df = 1; $P = 0.7064$) or survival benefit at an early time point (Gehan-Breslow-Wilcoxon test; chi-square = 0.4920, df = 1, $P = 0.4830$) was observed in *Drosophila* treated with LGG prior to 10 μM CP exposure (Fig. 6C). Together, these

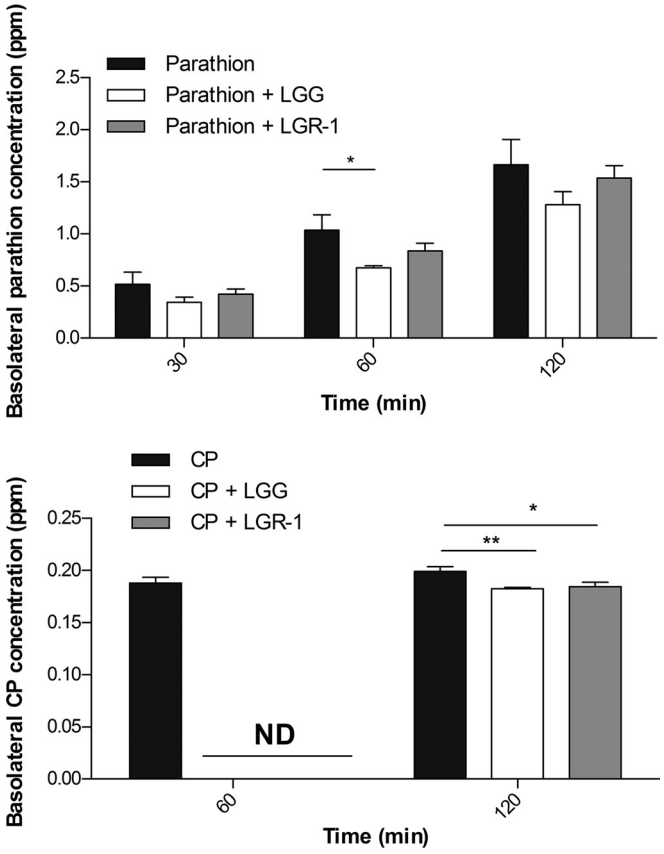


FIG 5 LGG and LGR-1 reduced the absorption of organophosphate pesticides in a Caco-2 Transwell model. Caco-2 cells, differentiated into small intestinal-like epithelium on Transwells, were exposed apically to 100 μM parathion or chlorpyrifos with or without 10^9 CFU/ml LGG or LGR-1. The basolateral compartment was kinetically analyzed for parathion or CP. Data displayed are means ± standard deviations of results from 4 independent experiments. *, $P < 0.05$; **, $P < 0.01$.

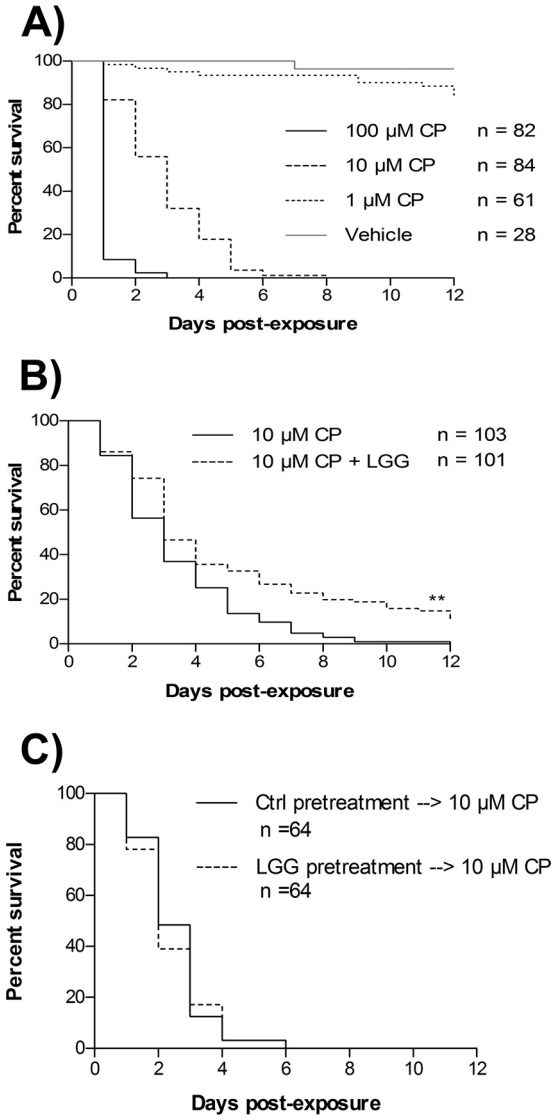


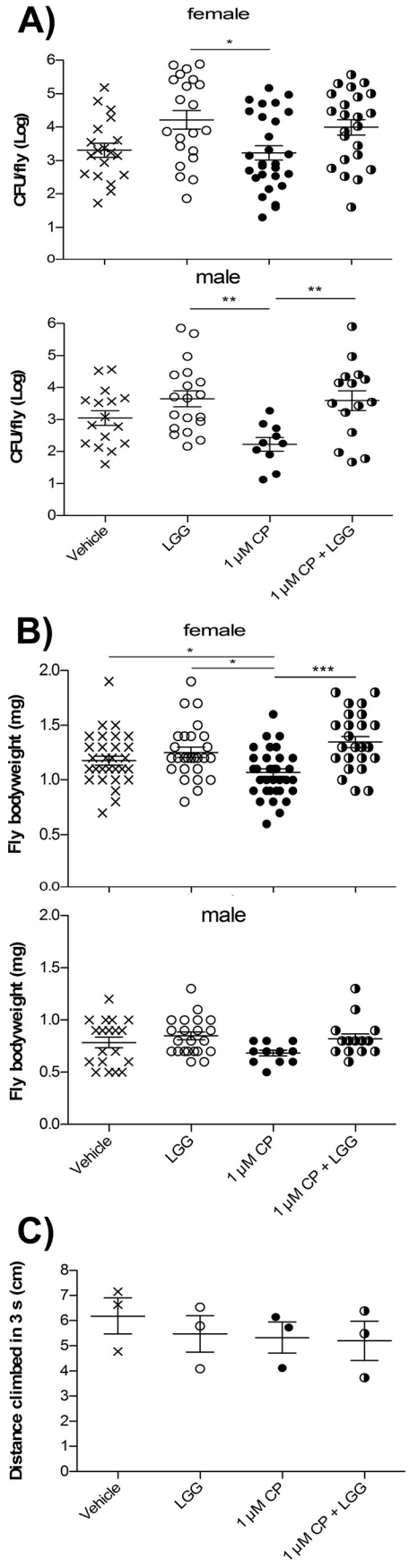
FIG 6 *Lactobacillus rhamnosus* GG supplementation reduces the toxicity of chlorpyrifos to *Drosophila melanogaster*. (A) Survival curves for freshly eclosed *Drosophila melanogaster* flies exposed to media containing 1, 10, or 100 μM CP or vehicle control. (B) Survival curves for freshly eclosed *Drosophila melanogaster* flies exposed to media containing 10 μM CP with or without simultaneous supplementation with live LGG. (C) Survival curves for freshly eclosed *Drosophila melanogaster* flies exposed to LGG or vehicle for 3 days and then subsequent exposure to media containing 10 μM CP. Data displayed are from at least 3 independent experiments. **, $P < 0.01$.

results suggest that LGG likely interacts directly with CP to mitigate CP-induced toxicity.

The direct effect of LGG supplementation on *Drosophila* microbiota composition and physiological responses following 3 days of exposure to 1 μM CP (a concentration that is not acutely lethal) were further characterized. Since lactobacilli are the dominant member of the *Drosophila* microbiota, lactobacilli were enumerated from *Drosophila* supplemented with LGG with or without simultaneous exposure to 1 μM CP. Both female and male *Drosophila* flies treated with LGG had significantly higher levels of lactobacilli than did *Drosophila* treated with 1 μM CP alone (Fig. 7A). Although 1 μM CP treatment did not significantly reduce *Drosophila* lactobacillus levels, they were notably lower in male flies and appeared to follow a bimodal response in females (some reduced, some unaffected) exposed to CP compared to vehicle controls. Furthermore, female *Drosophila* flies exposed for 3 days to medium containing 1 μM CP and LGG were significantly rescued ($P < 0.05$) from CP-mediated reductions in body weight (Fig. 7B). There was an insignificant trend toward similar body weight rescue in male flies. Moreover, since CP impacts insect locomotion due to exacerbated cholinergic stimulation, the ability of LGG supplementation to rescue *Drosophila* motor deficits following 3 days of 1 μM CP challenge was assessed in negative geotaxis assays. However, no differences in *Drosophila* locomotion (distance climbed after 3 s) were observed among the treatment groups of vehicle, LGG, 1 μM CP, and LGG with 1 μM CP (Fig. 7C). Together, these results suggest that (i) LGG supplementation can enhance the *Drosophila* microbiota with lactobacilli, and (ii) low-dose (1 μM) CP may cause developmental, rather than motor, deficits that can be rescued by LGG supplementation.

DISCUSSION

Lactobacillus strains were found to grow in medium containing high levels of parathion and CP. Since the commonly used probiotic LGG and LGR-1 strains displayed unaltered and altered



growth, respectively, in the presence of organophosphate pesticide, we further examined their activity. We found that the LGG hydrolase WP_014569076.1 was not responsible for organophosphate pesticide degradation. Although genetic cloning was not performed to definitively demonstrate that LGG_RS02045 was nonfunctional, LGG was not able to hydrolyze CP while *E. coli* BL21(DE3)(pET20b/*PTE*) was able to do so. Furthermore, heat-killed LGG had the same capability to sequester parathion and CP from solution as live LGG. This was also found with LGR-1 and suggests that metabolic activity is not required for bacterial organophosphate sequestration in these strains. Thus, unlike the findings reported by Cho et al. (38), we were unable to demonstrate degradation of the organophosphate pesticides parathion and CP by strains LGG and LGR-1. Differences in our experimental approach compared to the use of fermentation in organophosphate-contaminated dairy food products may explain this finding (29, 30). We suspect that the higher temperatures (42°C compared to 37°C) and acidic conditions present in the other *in vitro* fermentation studies promoted more rapid pesticide breakdown.

The finding of lactobacillus binding to organophosphate pesticides is congruent with reports of similar interactions with environmental toxins, such as aflatoxin (24, 25, 39), paralytic shellfish toxins (37), and metals (38–43). LGG had a better ability to retain CP sequestration—as measured by CP levels retained in bacterial pellets—from solution than LGR-1. This was also found in a Caco-2 Transwell model that simulated organophosphate absorption in the small intestine. This finding was surprising, given the genetic similarity between strains. One major difference between LGG and LGR-1 is that LGR-1 has a unique exopolysaccharide biosynthesis cluster (unpublished data). The abilities of lactobacillus-derived exopolysaccharide to bind toxins such as lead (44), cadmium (45), and aluminum (45) have been described previously. Furthermore, sediment microbe-produced exopolysaccharide has been shown to strongly interact with CP (46, 47). It is interesting to speculate that differences in bacterial exopolysaccharide may confer bacterium-organophosphate binding phenotypes in lactobacilli.

Results with *Drosophila* suggest that dietary supplementation of transitory bacteria can be important for conferring host benefits that are not present within the commensal microbiota. Specifically, prolonged interaction between CP and LGG may lead to increased survival. Unlike *Drosophila* exposed simultaneously to CP and LGG, those treated with LGG prior to pesticide exposure did not exhibit a survival benefit. These findings further support our hypothesis that LGG supplementation prevents downstream organophosphate pesticide toxicity by preventing absorption. Admittedly, we cannot completely rule out the possibility that LGG exposure results in priming of host detoxification pathways rele-

FIG 7 *Lactobacillus rhamnosus* GG supplementation promotes body weight gain and lactobacillus enhancement in chlorpyrifos-exposed *Drosophila* but does not affect locomotion. Freshly eclosed *Drosophila melanogaster* flies were treated for 3 days with media containing vehicle, 1 μM CP, LGG, or 1 μM CP plus LGG. (A) Surface-sterilized *Drosophila* flies were homogenized and drop plated on MRS agar, and the CFU per *Drosophila* fly were enumerated. (B) *Drosophila* body weights were determined. (C) *Drosophila* locomotion was tested in a negative geotaxis assay in which average distance climbed after 3 s (of 15 to 25 flies) in 5 replicate experiments was quantified. Data displayed are means ± standard errors of the means from at least 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

vant to organophosphate pesticides. Results from Kamaladevi et al. (48) suggest that the survival benefit experienced by CP-challenged *Caenorhabditis elegans* supplemented with *L. casei* may be due to upregulation of host phase II detoxification genes. However, this does not appear to be the mechanism of LGG-mediated prosurvival in *Drosophila* lethally challenged with CP (this study).

LGG supplementation was able to significantly rescue weight loss in female flies exposed to 1 μM CP. This trend was also observed in male flies; however, findings in males were not significant. The outcome corresponded with significantly increased lactobacillus CFU per fly after treatment with LGG relative to findings with CP treatment alone. *Lactobacillus* is the dominant genus in the *Drosophila* microbiota and is important for many host physiological processes (49, 50). *Lactobacillus plantarum* isolated from *Drosophila* has been shown to promote host growth in nutrient-depleted media via improved protein assimilation (50). Thus, similar to the findings reported by Blum et al. (51), we have shown that human probiotic LGG may be capable of conferring beneficial effects to host flies in a similar fashion as the native microbiota.

In summary, this study has shown that the commonly used probiotic organisms LGG and LGR-1 are able to bind, but not metabolize, organophosphate pesticides and reduce intestinal absorption *in vitro*. Furthermore, LGG reduced mortality and growth deficits in *Drosophila* exposed lethally and subchronically to CP, respectively. This work expands upon our previous study's findings, which demonstrated that *L. rhamnosus* supplementation could reduce heavy metal bioaccumulation in pregnant Tanzanian women (23). Transitory food-grade bacteria have the potential to act like a nonspecific "sponge"—absorbing toxins and reducing their uptake by the host. This approach holds promise for supplementing human, livestock, or apiary foods with probiotic microorganisms and reducing downstream toxicity from organophosphate pesticides.

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