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Probiotic *Escherichia coli* Nissle 1917 inhibits bacterial persisters that survive fluoroquinolone treatment

Patricia J. Hare[1,2], **Hanna E. Englander**[1,3], **Wendy W. K. Mok**[1]

[1]Department of Molecular Biology & Biophysics, UCONN Health, Farmington, Connecticut, USA

[2]School of Dental Medicine, UCONN Health, Farmington, Connecticut, USA

[3]Department of Physiology & Neurobiology, University of Connecticut, Storrs, Connecticut, USA

Abstract

Aims: Bacterial persisters are rare phenotypic variants in clonal bacterial cultures that can endure antimicrobial therapy and potentially contribute to infection relapse. Here, we investigate the potential of leveraging microbial interactions to disrupt persisters as they resuscitate during the post-antibiotic treatment recovery period.

Methods and Results: We treated stationary-phase *E. coli* MG1655 with a DNA-damaging fluoroquinolone and co-cultured the cells with probiotic *E. coli* Nissle following antibiotic removal. We found that *E. coli* Nissle reduced the survival of fluoroquinolone persisters and their progeny by over three orders of magnitude within 24 h. Using a bespoke H-diffusion cell apparatus that we developed, we showed that *E. coli* Nissle antagonized the fluoroquinolone-treated cells in a contact-dependent manner. We further demonstrated that the fluoroquinolone-treated cells can still activate the SOS response as they recover from antibiotic treatment in the presence of *E. coli* Nissle and that the persisters depend on TolC-associated efflux systems to defend themselves against the action of *E. coli* Nissle.

Conclusion: Our results demonstrate that probiotic bacteria, such as *E. coli* Nissle, have the potential to inhibit persisters as they resuscitate following antibiotic treatment.

Significance and Impact of the Study: Bacterial persisters are thought to underlie chronic infections and they can lead to an increase in antibiotic-resistant mutants in their progenies. Our data suggest that we can leverage the knowledge we gain on the interactions between microbial strains/species that interfere with persister resuscitation, such as those involving probiotic *E. coli* Nissle and *E. coli* MG1655 (a K-12 strain), to bolster the activity of our existing antibiotics.

Correspondence Wendy W. K. Mok, UCONN Health, Department of Molecular Biology and Biophysics, 263 Farmington Ave., Farmington, CT, 06032, USA mok@uchc.edu.
Present address Hanna E. Englander, Cutaneous Microbiome & Inflammation Section, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, Maryland, USA

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

CONFLICT OF INTEREST

No conflict of interest was declared.

Keywords

antibiotic treatment failure; *E. coli* Nissle; fluoroquinolones; microbial interference; persisters; probiotics

INTRODUCTION

Bacteria can deploy diverse strategies to overcome the plethora of stresses that they encounter in their environment, including those associated with the antibiotics that we currently use for infectious disease control (Brown & Wright, 2016; Davies & Davies, 2010). Bacterial pathogens that are resilient to our existing antibiotics impose a heavy global public health burden, one that claims over 750,000 lives worldwide annually (Centers for Disease Control and Prevention, 2019). This toll is projected to mount in the next decades if we fail to develop more effective infection control and treatment strategies (O'Neill, 2014).

Bacterial survival in the face of antibiotics is not solely dependent on their ability to acquire mutations that confer drug resistance. In genetically clonal cultures, subpopulations of bacteria that are coined persisters can reversibly enter into a growth-inhibited and/or metabolically repressed state where they are more tolerant to bactericidal antibiotics (Brauner et al., 2016; Pontes & Groisman, 2019; Shan et al., 2017). Once antibiotic therapy ceases, these persisters can resume growth and potentially contribute to infection relapse (Fauvart et al., 2011; Van den Bergh et al., 2017). As progenies derived from surviving persisters can adapt to antibiotic exposure, antibiotic persistence has also been shown to accelerate the evolution towards heritable resistance (Barrett et al., 2019; Byrd et al., 2021; Levin-Reisman et al., 2017; Liu et al., 2020).

The majority of bactericidal antibiotics in our arsenal are less efficacious towards nonreplicating bacteria compared with their growing counterparts (Eng et al., 1991; McCall et al., 2019; Zheng et al., 2020). Yet, at infection sites, bacteria are often exposed to conditions such as nutrient limitation, bacteriostatic agents and host defences that can limit growth (Blanc-Potard & Groisman, 2021; Kwan et al., 2013; Mok & Brynildsen, 2019; Rocha-Granados et al., 2020). These stresses can also enrich persisters that form in response to environmental triggers (Balaban et al., 2019; Brauner et al., 2016). Previously, our group and others demonstrated that some fluoroquinolones—a class of antibiotics that inhibits type II topoisomerases in bacteria and encompasses a number of compounds on the World Health Organizations list of essential medicines—can kill over 90% of slow–/non-growing bacteria (Barrett et al., 2019; Byrd et al., 2021; Drlica & Zhao, 1997; Mok & Brynildsen, 2018; Völzing & Brynildsen, 2015; WHO, 2019). Persisters from nonreplicating cultures are not exempt from fluoroquinolone-induced DNA damage (Völzing & Brynildsen, 2015). In fact, their resuscitation hinges on the activation of DNA repair enzymes following antibiotic removal (Völzing & Brynildsen, 2015). Furthermore, environmental conditions present during this post-fluoroquinolone treatment recovery period can affect the coordination of DNA repair and persister survival (Mok & Brynildsen, 2018). These findings suggest that there is a window of opportunity to sabotage the persister resuscitation programme

following the termination of antimicrobial therapy to reduce the burden of recurrent infections.

Here, we ask whether we can leverage the action of probiotics and the vulnerability of fluoroquinolone persisters originating from stationary phase cultures to sabotage persisters attempting to recover from antibiotic treatment. Probiotic bacterial strains/species can exert beneficial or therapeutic activities on their hosts, including bolstering host defence and reducing inflammation from pathogens (Gibson et al., 2017; Hill et al., 2014; Swanson et al., 2020). Specifically, we focus on probiotic *E. coli* Nissle 1917 and interrogate its effect on persister survival and DNA repair during the post-fluoroquinolone treatment period. *Escherchia coli* Nissle was isolated from a soldier who remained healthy during a dysentery outbreak in a German field hospital at the height of World War I (Sonnenborn, 2016). In the ensuing decades, *E. coli* Nissle has been incorporated as the active strain in Mutaflor, a probiotic sold in Europe, Canada and Australia for the prevention and management of gastrointestinal infections and disorders (Sonnenborn, 2016). This probiotic has been shown to stimulate the production of antimicrobial peptides, improve barrier function and enhance the immune response in host tissues (Rund et al., 2013; Schlee et al., 2007). *Escherichia coli* Nissle can secrete an array of antimicrobial molecules and enzymes, including iron-sequestering siderophores, bactericidal microcins and proteases, which enables this probiotic strain to compete with other *E. coli* strains as well as related Enterobacteriaceae (Fang et al., 2018; Sonnenborn, 2016). Although *E. coli* Nissle's antagonistic activity against its close relatives may seem counterintuitive, its inhibitory actions could serve to prevent pathogenic *E. coli* strains from invading its ecosystem. Close-range antagonistic interactions have also been shown to promote spatial segregation within some bacterial communities and preserve the diversity of its species or strains; in turn, this ecological diversity can strengthen the community's resilience to stressful perturbations (García-Bayona & Comstock, 2018). Beyond secreted antimicrobial agents, *E. coli* Nissle expresses an intact tripartite contact-dependent inhibition (CDI) system that can deliver a CdiA toxin to inhibit competing strains (Chen et al., 2018; Virtanen et al., 2019). In this investigation, we asked whether these antagonistic behaviours could be used to pit *E. coli* Nissle against a related strain, *E. coli* MG1655 and prevent persister recovery and regrowth following fluoroquinolone treatment. We show that while the fluoroquinolone persisters are able to engage in DNA repair as they recover in the presence of *E. coli* Nissle, their survival is decreased by over three orders of magnitude after 24 h in contact with the probiotic strain.

MATERIALS AND METHODS

Bacterial strains and strain construction

Escherichia coli MG1655, *E. coli* Nissle and their derivatives that were used in this study are listed in Table S1. *Escherichia coli* MG1655Δ*ompC*, Δ*ompF* and Δ*acrB* mutants were generated by P1 phage transduction using strains from the Keio Collection (*E. coli* BW25113Δ*ompC*::KanR, BW25113Δ*ompF*::KanR and BW25113Δ*acrB*::KanR) as donors and *E. coli* MG1655 as the recipient (Baba et al., 2006; Thomason et al., 2007). The *E. coli* MG1655Δ*tolC* mutant was constructed using the Datsenko–Wanner method as described previously (Byrd et al., 2021; Datsenko & Wanner, 2000). We also constructed

a chloramphenicol (Cam)-resistant *E. coli* Nissle derivative in which the chloramphenicol resistance marker is knocked into the *araBAD* locus using the Datsenko–Wanner method. Successful deletion of these genes was confirmed by colony PCR or Sanger sequencing using the primers listed in Table S2. The *recA* fluorescent transcriptional reporter plasmid (cloned into pUA66) and the plasmid used for the complementation of *tolC* in the deletion mutant (cloned into pBAD33) are described in Table S3 (Byrd et al., 2021; Völzing & Brynildsen, 2015).

Culture media and antibiotics

Unless otherwise indicated, the culture media components, chemicals and antibiotics used in this study were purchased from Fisher Scientific or Sigma-Aldrich. Luria–Bertani (LB) media, LB agar and Gutnick-glucose media were prepared and sterilized as described previously (Byrd et al., 2021). To select for transformants and transductants, 50 μg ml^{-1} of kanamycin (Kan; prepared as a 50 mg ml^{-1} stock) or 25 μg ml^{-1} of Cam (prepared as a 25 mg ml^{-1} stock) were used. For persister assays, cells were treated with 5 μg ml^{-1} of levofloxacin (Levo), which was prepared as a 5 mg ml^{-1} stock solution in MilliQ water and titrated with 1 mol l^{-1} NaOH until soluble. Levo and Kan were filter-sterilized using 0.22 μm filters before they were added to the media. Stock solutions of Cam were prepared in 200-proof ethanol and they were added directly to culture media following solubilization.

Measuring Levo persistence

Escherichia coli MG1655 or *E. coli* Nissle derivatives were inoculated into 2 ml of LB from −80°C frozen stocks and were cultured at 37°C, shaking at 250 rpm, for 4 h. The cells were then diluted 200-fold in 25 ml of Gutnick-glucose and cultured for 16 h. Following overnight growth, the optical density at 600 nm (OD_{600}) of each culture was measured and 500 μl of cells was collected for serial dilution and colony forming unit (CFU) enumeration. The remaining culture was treated with 5 μg ml^{-1} of Levo for 5 h, a treatment duration that has been shown to leave persisters as the only culturable cells in the population (Byrd et al., 2021). At designated times, 500 μl of each culture was collected for CFU enumeration, where cells were pelleted by centrifugation at 21,000 *g*, 450 μl of supernatant was removed, and the pellets were resuspended with 450 μl of phosphate-buffered saline (PBS). The wash step was repeated to reduce residual Levo to sub-minimum inhibitory concentration (MIC) levels. The cells were then subjected to 10-fold serial dilutions in PBS and 10 μl of each dilution was plated on LB agar. Surviving persisters that form colonies on the plates were enumerated after they were incubated at 37°C for 16 h.

Persister resuscitation in the presence of *E. coli* Nissle

To enable the selection of Levo persister progenies from a co-culture with *E. coli* Nissle, MG1655 derivatives that harbour a Cam (MO-CmR) or Kan resistance marker (MO001) were used (Mok et al., 2015; Orman & Brynildsen, 2013). To assess the impact of *E. coli* Nissle on the recovery of *E. coli* MG1655 or its derivatives following Levo treatment, persistence assays were carried out as described above. In these experiments, cells that were cultured to stationary phase and treated with sterilized water were incorporated as treatment-free controls. Following 5 h of Levo treatment, we collected cells to enumerate survivors in each culture to ensure that Levo treatment resulted in the killing of non-persisters.

At the same time, 1 ml of each culture (~1–2 × 10^9 cells) was collected and pelleted by centrifugation at 21,000 *g*. After removing 900 μl of supernatant, the pellets were resuspended in 900 μl of PBS. PBS washes were repeated and all of the supernatant was removed after the second wash. Cells in these pellets were recovered in 1 ml of LB plus either *E. coli* Nissle or wild-type *E. coli* MG1655, which served as a negative control.

Escherichia coli Nissle and its derivatives that were added to resuscitating Levo persisters were inoculated from −80°C frozen stocks into 2 ml of LB, where they were grown at 37°C with shaking at 250 rpm for 20–24 h. Following overnight growth, OD_{600} of the cultures were measured before 1 ml of each culture was pelleted by centrifugation and resuspended in 1 ml of LB. Assuming that 1 OD_{600} unit corresponds to ~8.8 × 10^8 cells per ml (Sezonov et al., 2007), we added volumes of cultures that yielded approximately 10^7, 10^8 or 10^9 *E. coli* Nissle cells to the Levo-treated *E. coli*. Wild-type *E. coli* MG1655 served as the negative control in these experiments and was cultured and prepared following the same procedures. At designated times, 10 μl of each culture was collected for serial dilution and plating on LB-Cam agar (to select for MO-CmR) or LB-Kan agar with 1 mmol l^{-1} IPTG (to select for MO001, which harbours a lactose-inducible copy of the Kan resistance marker). The agar plates were incubated at 37°C for 16 h before CFUs were enumerated.

In experiments involving membrane-bound protein deletion mutants of *E. coli* MG1655, the mutants were selected on LB-Kan agar. To complement the *tolC* deletion, *E. coli* MG1655Δ*tolC*::KanR was transformed with pBAD33 or pBAD33 with *tolC* expressed from its native promoter. These strains were cultured in LB-Cam media then diluted into Gutnick-glucose with Cam. After 16 h, the cultures were treated with Levo. Following Levo treatment and antibiotic dilution, the Levo-treated cells were co-cultured with *E. coli* Nissle or MG1655 bearing pBAD33 in LB-Cam, as described above. At designated times, 10 μl samples were collected for serial dilution and plating on LB-Kan agar to enumerate the survival of Δ*tolC*::KanR mutants harbouring either the pBAD33 control or the *tolC* complementation plasmid.

Impact of *E. coli* MG1655 on *E. coli* Nissle persister resuscitation

To investigate whether *E. coli* MG1655 affects *E. coli* Nissle persisters, we performed persister assays with a Cam-resistant derivative of *E. coli* Nissle following the protocol described above. After 5 h of Levo treatment, cells were collected and washed before being co-cultured with 10^7 or 10^8 *E. coli* MG1655 cells. Levo-treated *E. coli* Nissle::CmR was also co-cultured with wild-type *E. coli* Nissle as a negative control in these experiments. At designated times, 10 μl of each culture was collected for serial dilution and plating on LB-Cam agar. CFUs were enumerated after 16 h of incubation at 37 °C.

Contact-dependence of *E. coli* Nissle action

To determine whether the inhibition of Levo-treated cells by *E. coli* Nissle was dependent on direct cell–cell contact, we designed a glass H-diffusion cell that was custom-made to our specifications by Mr. Daryl Smith at the Yale University Scientific Glassblowing Laboratory. This apparatus permits the sterile co-culture of the two strains and dynamic exchange of diffusible molecules, while preventing cell migration between the two chambers. Two rubber

gaskets and a Pall SUPOR polyethersulfone (PES) membrane with 0.22 μm pores (Pall Inc.) were positioned in the flange joint connecting the two chambers ("arms") of the H-diffusion cell. This PES membrane is metabolite-permeable, but bacteria-impermeable. The connection of the H-diffusion cell was sealed with DuraSeal autoclavable laboratory stretch film (Fisher Scientific) and secured with a Delrin Keck Joint Clip for spherical joints (ChemGlass Life Sciences). The chamber openings were capped with two test tube lids. The entire unit was sterilized by autoclaving before use.

We confirmed that two populations of cells introduced into each arm of the H-diffusion cell would remain segregated throughout the course of our experiment by inoculating 5 ml of exponentially growing *E. coli* MO001 (Kan resistant) and *E. coli* MO-CmR (Cam resistant) into each arm of the device. At 0, 6 and 24 h post-inoculation, 10 μl of cells were collected from each arm, serially diluted and the dilutions were spotted onto LB-Kan agar with IPTG (to select for *E. coli* MO001) and LB-Cam agar (to select for *E. coli* MO-CmR). Colony formation on each set of plates was enumerated after 16 h of incubation at 37°C.

To assess the contact dependence of the interaction between Levo-treated cells and *E. coli* Nissle, *E. coli* MO-CmR was cultured overnight then subjected to 5 h of Levo treatment. Then, 5 ml of culture was collected by centrifugation at 21,000 *g*, washed twice with PBS to reduce Levo to sub-inhibitory concentrations as described above, and resuspended in 5 ml of LB broth. This population was inoculated into one arm of our custom-designed H-diffusion cell. The opposite arm of the H-diffusion cell was inoculated with 5 ml of LB containing 5×10^8 cells from an overnight culture of *E. coli* Nissle. At designated times throughout the post-Levo treatment recovery period, 10 μl of cells was collected for serial dilution, plating and CFU enumeration as described above.

We wanted to ensure that the PES membranes were not impeding the passage of large diffusible proteins or exosomes from *E. coli* Nissle that could mediate the inhibitory action on Levo-treated cells. To test this possibility, we inoculated *E. coli* Nissle and Levo-treated *E. coli* MO-CmR into independent arms of two different H-diffusion cells. We used two H-diffusion cells for each experimental replicate so that we could collect supernatant from the *E. coli* Nissle arm after 4 or 24 h co-culture. At the designated times, we collected ~5 ml *E. coli* Nissle from the H-diffusion cell, pelleted the cells by centrifugation at 21,000 *g* for 3 min, and collected the supernatant. We then recovered ~$1–2 \times 10^9$ Levo-treated MO-CmR in 500 μl of each supernatant mixed with 500 μl of two-fold concentrated LB to account for possible nutrient depletion of the spent media. We collected 10 μl of cells from each sample at designated times during the recovery period for serial dilution, plating on LB-Cam agar and CFU enumeration. If killing of the Levo-treated cells is not observed in the presence of *E. coli* Nissle supernatant, we can conclude that the inhibition of Levo persisters by *E. coli* Nissle is likely a contact-dependent phenomenon.

Activation of the SOS response in Levo-treated cells recovering in the presence of *E. coli* Nissle or MG1655

Escherichia coli MO-CmR bearing the *recA* transcriptional reporter plasmid, or the promoter-less pUA66 control, were inoculated into LB-Kan, diluted into Gutnick-glucose supplemented with Kan, and treated with Levo as described above. The cells were then

recovered in the presence of pUA66-bearing *E. coli* Nissle or *E. coli* MG1655 in 1 ml of LB-Kan. Three replicate aliquots of each culture were prepared, allowing us to collect samples at 0, 2, 4, 6 and 24 h post-Levo treatment. Before Levo administration, immediately after 5 h of Levo treatment, and at designated times during the post-antibiotic treatment recovery period, 500 µl of each sample was collected and pelleted by centrifugation at 21,000 *g* for 3 min. After removing the supernatant, the pellets were resuspended in 500 µl of 4% paraformaldehyde (PFA) in PBS to fix the cells. After incubating the samples on ice for 30 min, the samples were pelleted again, the supernatant was removed and the pellets were resuspended in 500 µl of PBS. The fixed cells were stored at 4°C until they were analysed by flow cytometry or microscopy.

For flow cytometry, the OD_{600} of the fixed cells was adjusted to ~0.01 in PBS and GFP-expressing cells in each sample were quantified using an LSRII flow cytometer with FACS DiVa (BD Biosciences) as described previously (Byrd et al., 2021). We analysed the cytometry data and plotted histograms using FlowJo (BD Biosciences). Cells were identified using forward and side-scattering parameters and the threshold for fluorescent cells was defined using a negative control which did not express GFP. We set a GFP-negative gate that captured >99% of events in the negative control; single cells in each sample with fluorescence intensities exceeding this gate were defined as fluorescent-positive.

To observe the cells under the microscope, the OD_{600} of each sample was adjusted to ~0.2 using PBS and 2 µl of each sample was spotted on 1% (w/v) agarose pads prepared with Bio-Rad molecular-grade agarose and MilliQ-purified water overlaid on a plain glass microscopy slide (Fisherbrand). The cells were then covered with a cover glass and sealed with clear nail polish to prevent evaporation before imaging using a Zeiss LSM 880 microscope with an oil-immersed 63× magnification objective. At least two fields of view were captured for each sample and the chosen images are representative of three biological replicates. The images were analysed using Zen Blue and ImageJ (Rueden et al., 2017).

Statistical analyses

At least three biological replicates were performed for each experiment unless otherwise noted. Graphs were plotted using GraphPad Prism and the error bars depict the standard error of the mean. Where indicated, statistical significance was determined using the Mann–Whitney *U*-test using GraphPad Prism. P-values for data points that are significantly different are presented in Table S4.

RESULTS

Escherichia coli Nissle inhibits the growth of fluoroquinolone persisters

Previously, it was reported that *E. coli* Nissle can inhibit the growth and biofilm formation of other *E. coli* strains and related Enterobacteriaceae species (Fang et al., 2018). Here, we asked whether this probiotic strain can limit the growth of *E. coli* fluoroquinolone persisters originating from stationary phase cultures as they recover from antibiotic treatment. For these experiments, we used an *E. coli* MG1655 derivative, MO-CmR, which bears a chromosomal copy of a chloramphenicol-resistance gene that can be used for selection

as our target (Figure 1a). We treated stationary-phase cultures of MO-CmR (the target) with Levo for 5 h (Figure 1b). Thereafter, we recovered the cells in LB in the presence of *E. coli* Nissle (the inhibitor) at a 1:1, 1:10 or 1:100 inhibitor:target ratio (Figure 1c). For comparison, stationary-phase MO-CmR that was not subjected to Levo treatment was co-cultured with *E. coli* Nissle at the same inhibitor:target ratios (Figure 1d). For our negative control, we replaced *E. coli* Nissle with *E. coli* MG1655 as the inhibitor strain.

We found that when Levo-treated *E. coli* MO-CmR and its treatment-free counterpart were co-cultured with *E. coli* Nissle at a 1:10 or 1:100 inhibitor:target ratio, culturability of the target significantly decreased after 6 h (Figure 1c,d). In contrast, *E. coli* MG1655 did not impact the growth and culturability of MO-CmR under any of the ratios that we tested. Interestingly, we did not observe any effect on culturability when *E. coli* Nissle was administered at a 1:1 ratio, perhaps because a higher abundance of *E. coli* Nissle could lead to nutrient exhaustion early in the co-culture period and limit target inhibition. For Levo-treated cells, a 1:10 ratio resulted in a 10-fold decrease in their culturability compared with populations challenged with a 1:100 ratio, indicating that the 1:10 ratio is optimal for target cell inhibition. Comparing the effect of the 1:10 Nissle:target cell ratio between treated versus untreated target cells, we observed that the survival of Levo-treated cells was significantly lower after 4 and 6 h co-culture with *E. coli* Nissle compared to the survival of the untreated cells co-cultured with Nissle. These results suggest that Nissle is even more effective against susceptible *E. coli* that have been treated with fluoroquinolones. Based on these findings, we used a 1:10 ratio of *E. coli* Nissle:Levo-treated cells in our subsequent experiments.

We further investigated whether the inhibitory effect of *E. coli* Nissle is unidirectional or if *E. coli* MG1655 could also inhibit the resuscitation of *E. coli* Nissle persisters. We found that *E. coli* MG1655 did not affect the culturability of Levo-treated *E. coli* Nissle as they recovered from treatment (Figure S1). These results suggest that *E. coli* MG1655 cannot reciprocally inhibit *E. coli* Nissle persisters.

Fluoroquinolone-treated *E. coli* activates the SOS response in the presence of *E. coli* Nissle

Upon finding that *E. coli* Nissle can inhibit the survival of Levo persisters as they recover from antibiotic treatment, we asked whether *E. coli* Nissle interferes with the molecular events that are critical to fluoroquinolone persister resuscitation. We and others previously reported that stationary-phase *E. coli* recovering from fluoroquinolone treatment induce the SOS response after the antibiotic had been diluted to sub-inhibitory concentrations, regardless of whether the cell succumbs to fluoroquinolone-induced DNA damage or persists (Barrett et al., 2019; Byrd et al., 2021; Mok & Brynildsen, 2018; Murawski & Brynildsen, 2021; Völzing & Brynildsen, 2015). Indeed, when SOS response genes, such as *recA, lexA* and *recB* are deleted or mutated, survival of fluoroquinolone persisted decreases by orders of magnitude (Völzing & Brynildsen, 2015). Furthermore, delaying *recA* expression as cells resuscitate following fluoroquinolone removal compromises persister survival (Mok & Brynildsen, 2018).

Because environmental triggers, such as nutrient availability, can modulate the timing of molecular events involved in post-treatment persister repair and growth resumption, we investigated the impact of *E. coli* Nissle on the coordination and activation of DNA damage repair in Levo-treated target cells (Mok & Brynildsen, 2018). We treated *E. coli* MO-CmR bearing a fluorescent reporter for *recA* expression with Levo and recovered the cells in the presence of *E. coli* Nissle or *E. coli* MG1655 as a negative control (Figure 2a). When we monitored *recA* expression for 6 h during the post-Levo treatment recovery period, we detected fluorescence in a comparable number of Levo-treated cells regardless of whether they recovered in the presence of *E. coli* Nissle or MG1655 (between 70% and 80% of cells from both populations were fluorescent after 2 h of recovery; Figure 2b). Further, we observed that cells co-cultured with *E. coli* Nissle or MG1655 had comparable levels of fluorescence, indicating that induction of the SOS response occurred at similar levels regardless of the inhibitor strain (Figure S2). When we examined these Levo-treated cells by fluorescence microscopy, we observed that, irrespective of the inhibitor strain, the majority of fluorescent target cells remained intact and formed filaments—a morphology that is frequently observed in *E. coli* undergoing the SOS response (Cohen & Barner, 1954; Huisman & D'Ari, 1981) (Figure 2c,d; Figure S3). Collectively, these results suggest that *E. coli* Nissle does not inhibit *E. coli* cells recovering from exposure to Levo by disrupting their activation of the SOS response.

Escherichia coli Nissle inhibits fluoroquinolone persisters in a contact-dependent manner

Escherichia coli Nissle has been shown to secrete siderophores and microcins that can inhibit competing entero-bacteria (Rund et al., 2013; Sonnenborn, 2016). It can also inhibit the growth of its competitors in a contact-dependent manner (Virtanen et al., 2019). To elucidate whether *E. coli* Nissle inhibits the growth and colony formation of *E. coli* recovering from Levo treatment in a contact-dependent or contact-independent manner, we designed an H-diffusion cell for co-culturing the two strains (Figure 3a,b; Figure S4). We found that when these two strains were co-cultured in the H-diffusion cell, *E. coli* Nissle's inhibitory effect was no longer observed (Figure 3c). However, the PES filter 220 nm pore size could potentially limit the diffusion of secreted extracellular vesicles from *E. coli* Nissle that range from 149 to 189 nm in diameter (Hong et al., 2019). To ensure that the PES filter does not impede extracellular vesicles or large globular proteins from mediating the inhibitory interaction, we collected supernatant from the *E. coli* Nissle arm of the H-diffusion cell after 4 and 24 h of co-culturing with Levo-treated *E. coli* MO-CmR (Figure S5A). The supernatant from the Nissle arm of the H-diffusion cell is expected to contain any potential inhibitory molecules that could not travel to the other arm. We then applied this supernatant to another population of Levo-treated *E. coli* and measured their survival over 24 h. We found that, consistent with populations that were co-cultured with *E. coli* Nissle in the H-diffusion cell, exposing Levo-treated cells to *E. coli* Nissle supernatant did not affect their survival as they recovered from antibiotic treatment (Figure S5B). From the results of these two experiments, we conclude that the inhibition of Levo persisters by *E. coli* Nissle is likely a contact-dependent phenomenon.

Escherichia coli recovering from Levo treatment depend on TolC-associated efflux pumps to defend against the inhibitory effects of *E. coli* Nissle

In *E. coli*, contact-dependent growth inhibition has been shown to be mediated by a β-barrel CdiB protein from the inhibitor strain, which can export and present a stick-like CdiA protein to its target (Aoki et al., 2005). When CdiA interacts with receptors on the surface of its neighbouring target cell, the effector that mediates the inhibitory effect in its C-terminal domain is cleaved and delivered into the target cell (Aoki et al., 2008). *Escherichia coli* strains have been found to harbour different classes of CdiA proteins, which are categorized based on the sequence homology of their receptor-binding domains. As outer membrane porins OmpF and OmpC have been shown to serve as receptors for one class of CdiA proteins, and the inner membrane transport protein AcrB has been implicated in the inhibitory activity of another class (Aoki et al., 2008; Virtanen et al., 2019), we asked whether these membrane-bound proteins are necessary for the interactions between *E. coli* Nissle and cells recovering from Levo treatment. Additionally, in Gram-negative bacteria such as *E. coli*, tripartite efflux pumps can mediate the expulsion of antibiotics and toxins from the cell (Li et al., 2015). A number of these efflux pumps, including the one that contains AcrB, have TolC as the outer membrane component. Therefore, we also asked whether efflux pumps containing TolC provide protection against the inhibitory determinant(s) from *E. coli* Nissle.

Compared with the Kan-resistant wild-type control, survival of the deletion mutants was a few fold lower following 5 h of Levo treatment, with decreases in the Δ*ompF* (eight-fold) and Δ*tolC* (five-fold) mutants being statistically significant (Figure 4a). We found that Levo-treated *E. coli* MG1655 lacking *ompF, ompC*, *acrB* and *tolC* remained sensitive to the effects of *E. coli* Nissle (Figure 4b-e). Indeed, when genes encoding these membrane-associated proteins were deleted, survival of the mutants was reduced compared with their wild-type counterpart following 24 h of co-culture with *E. coli* Nissle. By comparison, the survival of the mutants was comparable to wild-type *E. coli* when they were co-cultured with *E. coli* MG1655.

Of the four mutants that we tested, only the loss of *tolC* was found to result in a significant reduction in survival in the presence of *E. coli* Nissle in all of the time points that we sampled (Figure 4d). After 24 h of co-culture, survival of the Δ*tolC* mutant decreased 150-fold compared with the wild-type control. When *tolC* is expressed from its native promoter on pBAD33 in the deletion mutants, the complemented strains were no longer sensitive to the effects of *E. coli* Nissle (Figure 4e). Collectively, these data suggest that TolC offers susceptible *E. coli* strains protection from the contact-dependent inhibitory effects of *E. coli* Nissle as they recover from Levo treatment.

DISCUSSION

Antibiotic susceptibility is traditionally investigated in the monospecies context. Yet, in infection niches, pathogens seldom exist in isolation. In these polymicrobial communities, cross-feeding, crosstalk and exchange of secreted metabolites and enzymes between different bacterial strains and species have been found to affect the survival of individual constituents following exposure to antibiotics (de Vos et al., 2017; Tavernier et al., 2017,

2018). For instance, the Conlon group has shown that in infections involving *Pseudomonas aeruginosa* and *Staphylococcus aureus*—two species that often co-occur in wounds and in the lungs of cystic fibrosis patients—*P. aeruginosa* can secrete molecules such as rhamnolipids that sensitize *S. aureus* persisters to aminoglycoside drugs (Radlinski et al., 2017, 2019). Conversely, *S. aureus* can also secrete exoproducts that influence *P. aeruginosa* biofilm formation, metabolism and growth; but the impact of these molecules on the antibiotic susceptibility of *Ps. aeruginosa* remains to be elucidated (Zarrella & Khare, 2021). In our work, we demonstrate that we can leverage microbial interactions after antibiotic treatment to reduce persister survival as they attempt to resuscitate following fluoroquinolone treatment.

Our in vitro work implies that probiotic microbes can potentially work in conjunction with existing antibiotics to reduce the burden of recurrent infections after a course of antimicrobial therapy. We have designed an electro-chemical H-cell-inspired apparatus that can be used to interrogate the contact dependence of persistence-reducing interactions between additional probiotic-pathogen pairs. Compared with the use of cell-free conditioned media derived from a donor strain to investigate the impact of microbial interactions, this apparatus enables us to monitor the continuous crosstalk of the two bacterial strains/species and measure phenotypic changes in each population independently. Unlike commercial products like Trans-wells, this H-diffusion cell maintains spatially distinct cell populations with equal oxygenation, growth surfaces and media volumes. The H-diffusion cell is also reusable, which can reduce the cost and plastic waste associated with these experiments. Beyond *E. coli* Nissle, the anti-persister activity of additional probiotic bacterial strains awaits our investigation, and this H-diffusion cell can improve experimental workflows to facilitate these discoveries.

Escherichia coli Nissle is known to secrete enzymes and antimicrobial compounds, such as microcins, to antagonize its competitors but experiments carried out with our H-diffusion cell suggest that *E. coli* Nissle's effect on *E. coli* MG1655 derivatives recovering from Levo treatment is contact-dependent. Recently, contact-dependent inhibition (CDI) systems, which comprise a CdiB β-barrel protein that exports the toxic CdiA effector to its targets, were identified in *E. coli* Nissle (Chen et al., 2018). CdiA proteins that have been found in different *E. coli* strains have been classified based on sequence homology, and each class has been shown to interact with distinct receptors on its targets. *Escherichia coli* Nissle is predicted to carry a Class II CdiA, which can interact with outer membrane porins OmpF and OmpC of its target strains (Beck et al., 2016; Virtanen et al., 2019). Yet, when we deleted *ompF* or *ompC* from *E. coli* MG1655, Levo persisters originating from stationary-phase cultures remained susceptible to the inhibitory effects of *E. coli* Nissle. These data suggest that OmpF and OmpC are not required to mediate the inhibitory action of *E. coli* Nissle on fluoroquinolone persisters.

In *E. coli* strains that harbour Class I CdiA systems, AcrB, the inner membrane component of AcrAB-TolC tripartite multidrug efflux pumps, is implicated in target cell inhibition by CdiA (Aoki et al., 2008). Therefore, we would expect Δ*acrB* mutant target cells to be immune to the effects of *E. coli* Nissle if Class I CdiA were the conduit for its inhibitory action. However, similar to the Δ*ompF* and Δ*ompC* mutants, we observed that Δ*acrB*

mutants remained sensitive to the effects of *E. coli* Nissle. In fact, in some of the replicates, survival of the Δ*acrB* mutant was reduced compared with its wild-type counterpart. This led us to further test whether the efflux function of AcrB provides defence against *E. coli* Nissle. As the TolC outer membrane component of the AcrAB-TolC efflux pump is associated with other members of the resistance-nodulation-division (RND) class of multidrug efflux pumps, we deleted *tolC* from *E. coli* MG1655 and found that the Δ*tolC* mutant was significantly more susceptible to *E. coli* Nissle following Levo treatment. The precise molecular basis underlying the interaction between Levo-treated cells and *E. coli* Nissle will be the subject of future investigations. Nevertheless, as survival of persisters treated with certain antibiotics depends on the action of TolC-associated RND efflux pumps, our findings suggest that targeting efflux pump action can have dual function in bolstering the activities of certain antibiotics and probiotics like *E. coli* Nissle (Byrd et al., 2021; El Meouche & Dunlop, 2018; Pu et al., 2016).

In our experiments, we challenged fluoroquinolone persisters originating from slow–/non-growing cultures with *E. coli* Nissle after the antibiotic was removed. The administration of this regimen is not practical clinically, because the probiotic strain can only be introduced and allowed to grow after the antibiotic has reduced to a sub-inhibitory concentration. To facilitate the use of probiotics such as *E. coli* Nissle to interfere with the persister resuscitation program, one strategy could be to engineer probiotic strains that can withstand antibiotic treatment. While many bacterial toxin-antitoxin (TA) systems have been linked to phage defence (Goormaghtigh et al., 2018; Guegler & Laub, 2021; Harms et al., 2018), it has been demonstrated that the ectopic overexpression of toxins from TA systems can increase tolerance or persistence towards different antibiotic classes (Lemma & Brynildsen, 2021; Mok et al., 2015; Vázquez-Laslop et al., 2006). Given that *E. coli* Nissle has been found to have chromosomally encoded TA modules, the strain could be engineered to orthogonally express genes encoding its endogenous toxins and antitoxins (Xu et al., 2020). By this design, *E. coli* Nissle can potentially be programmed to accumulate toxins to tolerate antibiotic treatment, then it can be induced to accumulate antitoxins to reawaken in a timely manner and inhibit pathogens after the antibiotic drops below an inhibitory concentration.

To circumvent the use of antibiotic resistance markers that can be horizontally transferred to pathogens in engineering *E. coli* Nissle variants, TA genes can be introduced into auxotrophic *E. coli* Nissle strains, similar to the approach that Hwang and colleagues used (Hwang et al., 2017). This team introduced a genetic circuit harbouring genes encoding alanine racemases to complement deletions in their *E. coli* Nissle host, producing an antibiotic resistance marker-free strain that is capable of sensing, killing and disrupting biofilm formation in *P. aeruginosa* (Hwang et al., 2017). To further enhance the safety of using *E. coli* Nissle therapeutically, it was recently reported that its genotoxic activity stemming from the activation of colibactin can be uncoupled from its probiotic activities (Massip et al., 2019). The discovery of additional probiotic strains that can target bacterial persisters coupled with the use of synthetic biology to improve the performance and safety of these strains can potentially expand our repertoire of antibiotic adjuvants to diminish the burden of antibiotic treatment failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article or from the corresponding author, upon request.

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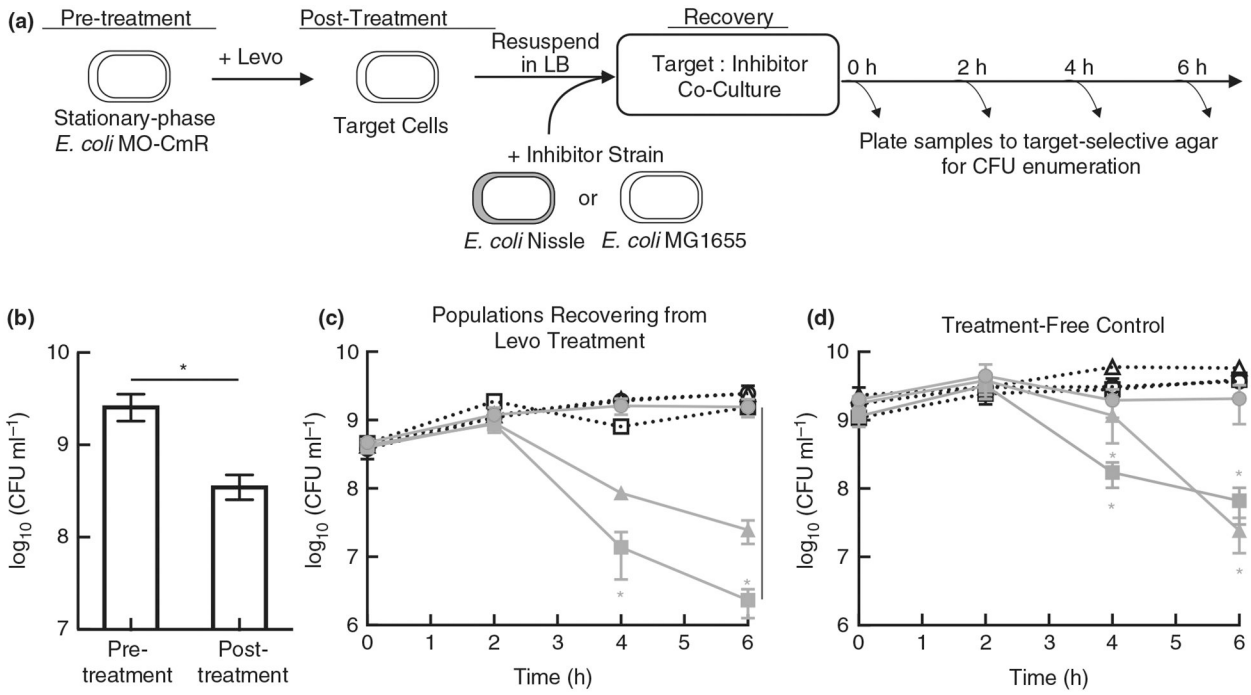

FIGURE 1.

Probiotic *Escherchia coli* Nissle inhibits the resuscitation of Levo persisters originating from slow-/non-growing cultures following antibiotic treatment. (a) Overview of experiment. (b) Treating stationary-phase *E. coli* cultures with Levo for 5 h reduced survival of the population by 10-fold. (c) Co-culturing Levo-treated stationary-phase *E. coli* MO-CmR (target strain) with *E. coli* Nissle (inhibitor strain) reduced Levo persister levels by 10-fold and 100-fold when the target:inhibitor ratios were 100:1 and 10:1, respectively. Co-culturing Levo-treated with *E. coli* Nissle at a 1:1 ratio or with *E. coli* MG1655 (control inhibitor strain) did not reduce persister survival under similar growth conditions. (d) When stationary-phase *E. coli* MO-CmR (target) that had not been exposed to Levo was co-cultured with *E. coli* Nissle (inhibitor), target:inhibitor ratios of 10:1 or 100:1 also reduced the survival of MO-CmR strain by 10-fold. For panels (b) and (c), target:inhibitor ratios are represented by (●) 1:1; (■) 10:1; (▲) 100:1. Filled grey symbols denote *E. coli* Nissle as the inhibitor; hollow black symbols denote *E. coli* MG1655 as the inhibitor. At least three biological replicates were performed for each experiment and error bars represent the standard error of the mean. In panel (b), the asterisk denotes statistically significant differences ($p \leq 0.05$) between colony counts before treatment and after 5 h of Levo treatment. In panels (c) and (d), the asterisks denote statistically significant differences ($p \leq 0.05$) in survival between cells co-cultured with *E. coli* Nissle and cells co-cultured with *E. coli* MG1655 at the same target:inhibitor ratios

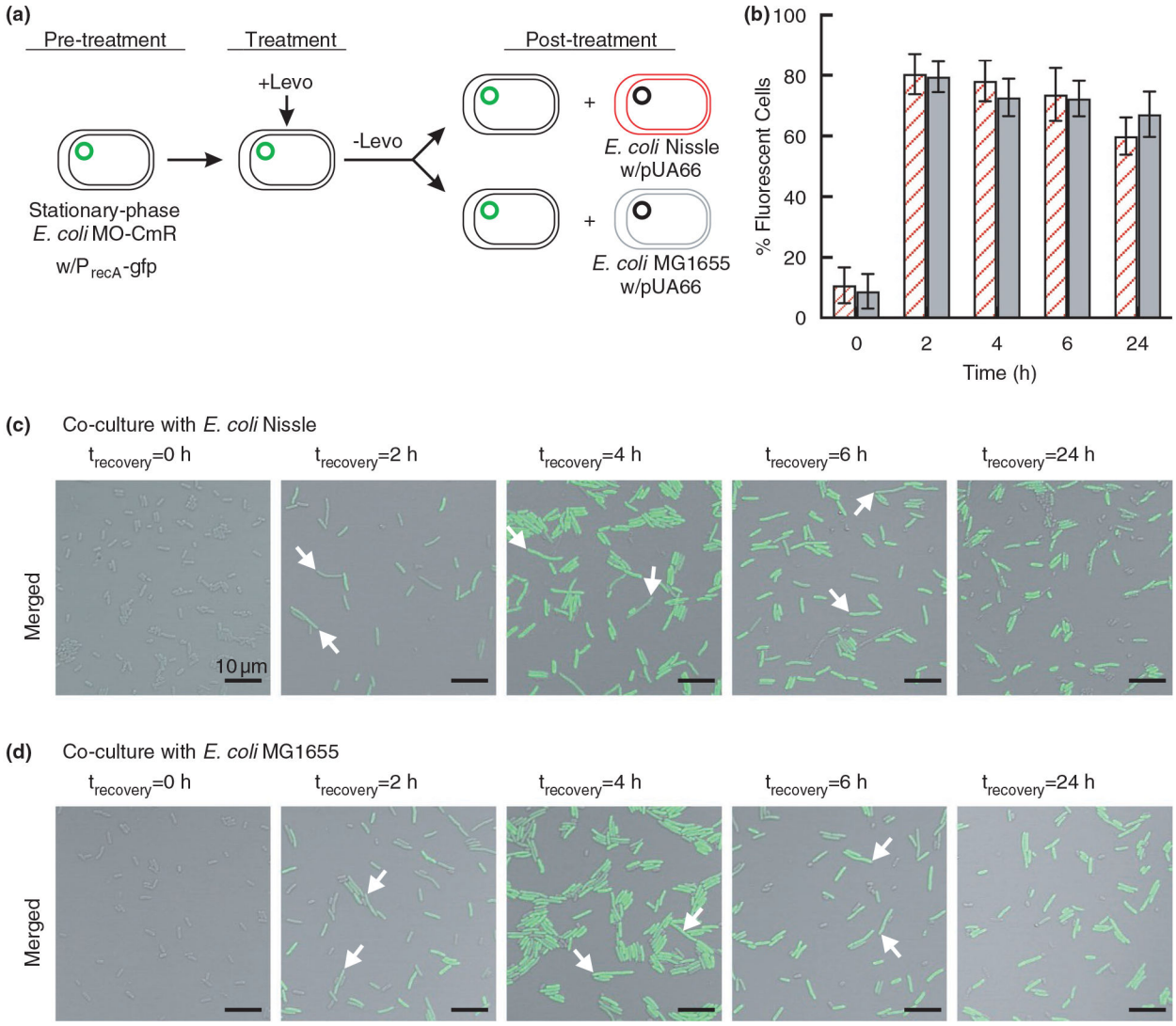

FIGURE 2.

Escherchia coli Nissle does not interfere with DNA repair in cells recovering from Levo treatment. (a) Experimental schematics: *E. coli* MO-CmR that harboured a P_{recA}-*gfp* reporter were treated with Levo and recovered in the presence of *E. coli* Nissle or *E. coli* MG1655. Cells were collected for flow cytometry and fluorescence microscopy analysis during the post-Levo treatment recovery period. (b) In co-cultures with *E. coli* Nissle or MG1655, 70%–80% of the target population induced *recA* expression within 2 h after inoculation into LB media with Kan. The hashed bars represent data from co-cultures with *E. coli* Nissle, whereas the filled grey bars represent data from co-cultures with *E. coli* MG1655. (c) Merged brightfield and fluorescence microscopy images of *E. coli* MO-CmR co-cultured with *E. coli* Nissle following Levo treatment show that the majority of fluorescent cells were filamentous (examples are indicated by white arrows). (d) Microscopy images of Levo-treated *E. coli* MO-CmR that were co-cultured with *E. coli* MG1655 during the post-antibiotic treatment recovery period show that many of the cells were also fluorescent and filamentous, indicating that they induced the SOS response. The flow

cytometry histograms and microscopy images shown here are representative of data from three biological replicates

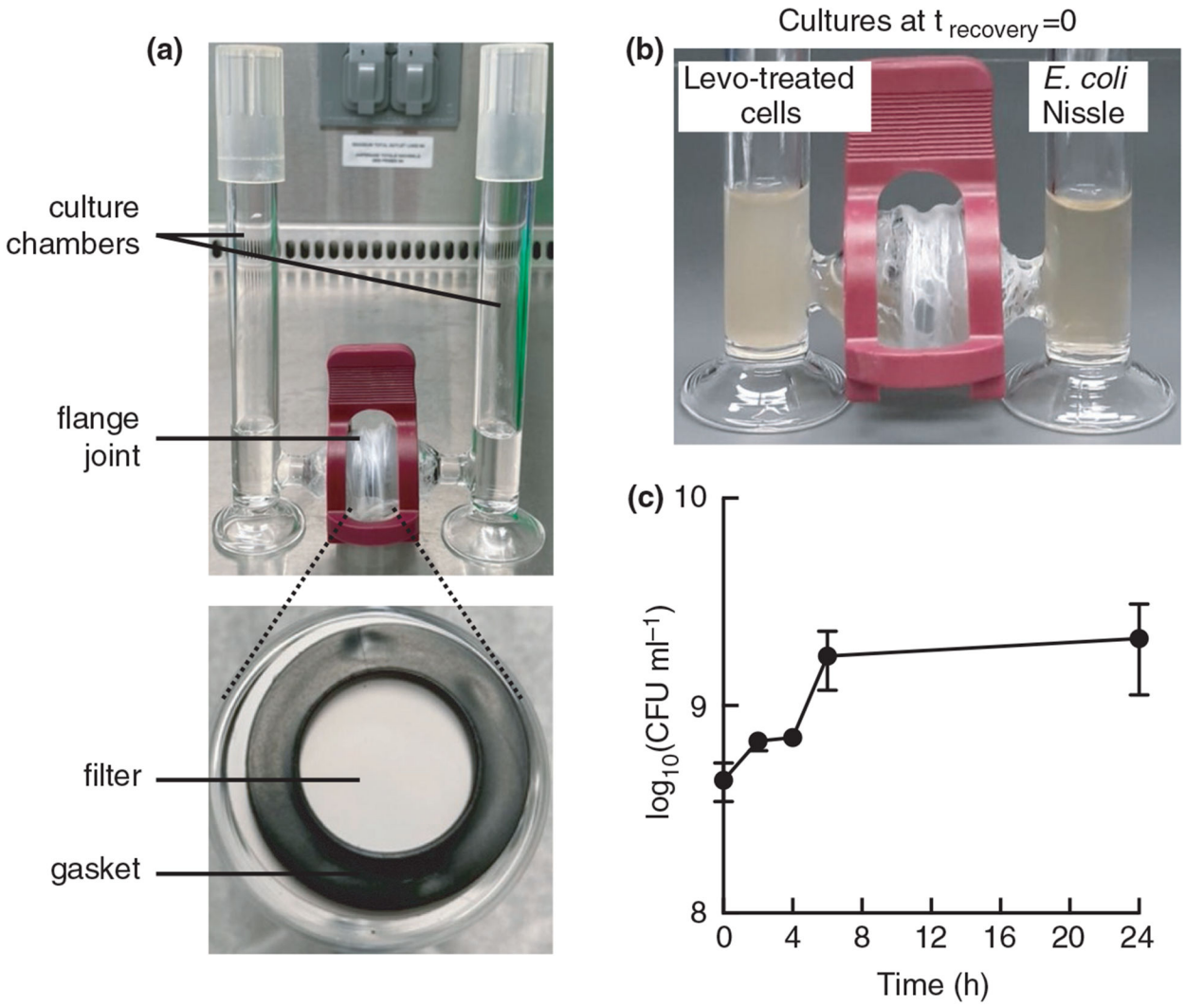

FIGURE 3.

Escherchia coli Nissle inhibits Levo-treated cells originating from stationary-phase cultures in a contact-dependent manner. (a) Design of the H-diffusion cell that enabled co-culture of *E. coli* Nissle and Levo-treated *E. coli* MO-CmR in a contact-independent manner. The two *E. coli* strains are separated by a PES filter with 0.22 μm pores that prevent bacterial migration. (b) At the onset of the experiment, Levo-treated *E. coli* MO-CmR was inoculated in one arm of the H-diffusion cell, whereas *E. coli* Nissle was inoculated in the other arm. (c) Recovery and growth of Levo-treated *E. coli* MO-CmR that was co-cultured with *E. coli* Nissle following fluoroquinolone removal. When the two strains were co-cultured in the H-diffusion cell, the probiotic strain no longer inhibited the target. The data points depict averages from three biological replicates and error bars represent the standard error of the mean

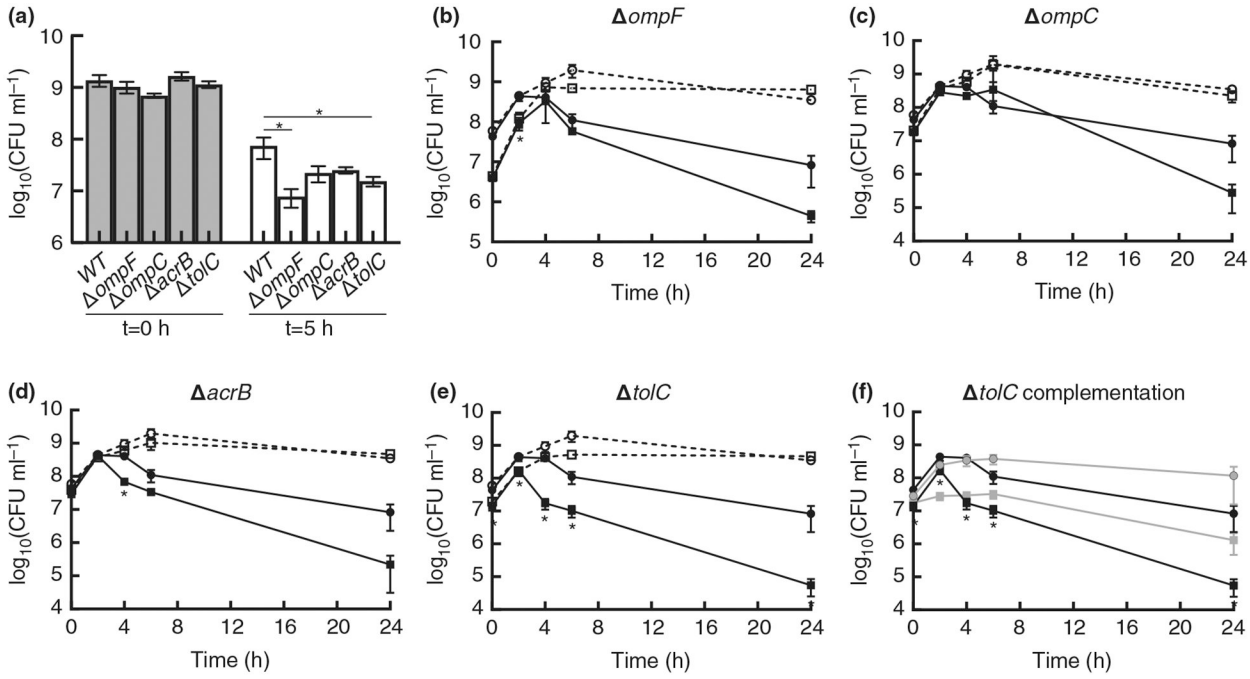

FIGURE 4.

Impact of deleting membrane-associated proteins on the susceptibility of Levo-treated cells to *Escherchia coli* Nissle. (a) Survival of *E. coli* MO001 (an *E. coli* MG1655 derivative that bears a Kan resistance marker) and mutants of *E. coli* MG1655 that lack *ompF*, *ompC*, *acrB* or *tolC* following 5 h of Levo treatment. Deletion of *ompF* (b), *ompC* (c) or *acrB* (d) did not significantly reduce survival of the Levo-treated mutants when co-cultured with *E. coli* Nissle during the post-fluoroquinolone treatment recovery period, as compared to wild-type MO001 plus *E. coli* Nissle. (e) Deletion of *tolC* significantly reduced survival after the cells had been treated with Levo and recovered in the presence of *E. coli* Nissle. For panels (b), (c), (d) and (e), (●) denotes MO001 target with *E. coli* Nissle inhibitor; (○) denotes MO001 target with *E. coli* MG1655 inhibitor; (■) denotes mutant target with *E. coli* Nissle inhibitor; and (□) denotes mutant target with *E. coli* MG1655 inhibitor. (f) Complementation of *tolC* expressed from its native promoter in pBAD33 protected the Δ*tolC* mutant from the inhibitory effects of *E. coli* Nissle. Each data set in panel (f) used *E. coli* Nissle inhibitor plus target strain (●) MO001, (■) Δ*tolC* mutant, (■) Δ*tolC* mutant with pBAD33, or (●) Δ*tolC* mutant with pBAD33::P_{tolC}-*tolC*. At least three biological replicates were performed for each experiment and error bars depict the standard error of the mean. Asterisks denote statistically significant differences ($p \leq 0.05$) between the survival of the mutant and the survival of the wild-type (*E. coli* MO001) counterpart