9. Future directions

In the body of work presented, we explored the response of *S*. Typhimurium to ciprofloxacin, optimised assays for high-content imaging of bacteria, and probed for genomic and phenotypic characteristics in *S*. Typhimurium ST313. While these studies produced some interesting observations, there are many additional avenues of exploration that would strengthen our understanding of *S*. Typhimurium and the bacterial response to ciprofloxacin and other stressors.

9.1 Further development of genotype-phenotype investigations

In Chapter 3, we developed methodologies for high-content imaging (HCI) of bacteria using the Opera Phenix imaging platform. While we have implemented a fairly robust imaging setup, there are many improvements and advances that could be made to our methods. Firstly, given the resolution of single-bacterium imaging on the Opera Phenix, it would be useful to study bacterial subcellular properties. One strategy for doing so would be to develop bespoke reporter bacterial stains, such as with novel combinations of LPS and O-antigen to study biofilm formation[569]. Additionally, isolates expressing GFP of specific reporters might be useful. In addition, the detection of flagella could also be important, possibly even to determine how well a given isolate might adhere to imaging plates[570]. Another way to better study bacterial subcellular properties would be to improve the analysis pipelines in the Perkin Elmer Harmony software. In our existing pipelines, we did not segment bacteria after classifying them as single cells, but it may be possible to delineate membrane thickness, localization of nucleic acids, and other properties, with more fine-tuning. Doing so might give us some insight into how bacterial subcellular morphologies change with perturbation or vary between organisms[571]. Finally, the next step in understanding bacterial population heterogeneity and temporal changes is to implement live-cell imaging using the Opera Phenix. An added complexity of doing this on bacteria alone is their motility, and live cell imaging of *Salmonella* has largely been done in the context of host cells[572]. Single-cell live bacteria imaging has been achieved on low-throughput confocal microscopes, and Varadarajan *et al.* describe a time-lapse microscopy protocol for *E. coli* using agarose gel pads, and a similar

protocol could be adapted for *Salmonella*[573]. Plochowietz *et al.* used a similar agarose gel pad method for imaging tRNA diffusion within live *E. coli*, illustrating the utility of single cell imaging for not only tracking individual bacteria over time but also visualising subcellular changes[574].

We used our existing fixed-cell imaging method in Chapter 4 to study the temporal response of *S*. Typhimurium to ciprofloxacin, and while we were able to observe changes in bacterial length over time in response to ciprofloxacin, there is far more to be investigated. One important assessment would be determining the most influential parameters influencing bacterial morphological changes and how those vary between treatments. Additionally, we were able to measure the distribution of bacterial length over time, but we did not distinguish subpopulations within each field. Given our findings regarding the different transcriptional responses of denser and less-dense bacteria upon ciprofloxacin exposure, it would be important to determine the heterogeneity of morphology in the population. Many hypotheses exist about the nature of bacterial heterogeneity in response to stress, and it remains unclear whether a given bacterial population is homogeneous prior to perturbation and diversifies its response or is initially heterogeneous and that subpopulations respond or expand differentially[575].

Sánchez-Romero and Casadesús demonstrated the role of differentially expressed efflux pumps and porins in a subpopulation of *S*. Typhimurium grown in nalidixic acid, revealing the importance of studying these[576]. It is not yet known whether these subpopulations can be morphologically distinguished at the single cell level, which could be investigated using single cell imaging and differentiating the bacterial populations through image analysis. Ideally, this would be performed using live cell imaging, which could additionally inform us about the relative growth and viability of individual cells. Sánchez-Romero and Casadesús used flow cytometry to differentiate between subpopulations, and it may be possible to use fluorescence activated cell sorting to separate these for whole genome and RNA-sequencing to determine mutational and adaptive resistance[576]. It has previously been demonstrated that bacterial efflux may increase due to gene duplications of *acrAB*, and measuring genetic and epigenetic changes in a subpopulation of ciprofloxacin-treated bacteria may help pinpoint which processes are responsible[577,578].

Furthermore, we did not link the phenotypes we observed regarding temporal growth to the genetic background of the bacteria measured. We observed that there were differences between

the two *S*. Typhimurium isolates measured—ST313 D23580 and ST34 VNS20081—but we did not perform a direct comparison of these two. Here, it would be informative to look at SNP differences between the two isolates and determine whether there are any that could explain the morphological changes we observed. It may also be relevant to compare the transcriptional response of subpopulations of the two bacterial isolates to understand the heterogeneity of response to ciprofloxacin across isolates.

In Chapter 7, we similarly used the Opera Phenix to screen a diverse set of 24 S. Typhimurium ST313 isolates in the presence of ciprofloxacin. There, we did attempt to connect the morphological differences between the isolates to their genetic backgrounds, although there is far more to be studied. To further develop this approach, it would be useful to screen a larger set of isolates. Doing so would tell us whether the variation we observed between isolates holds true across the phylogenetic tree of ST313. Van Puyvelde *et al.* have observed repeated phylogenetic substructures within *S*. Typhimurium ST313 lineage II, and it would be useful to understand how these phylogenetically distinct groups cluster morphologically, and whether there is convergent evolution towards a common phenotype (Van Puyvelde, personal communication)[176]. Moreover, MacKenzie *et al.* have recently studied biofilm formation in invasive nontyphoidal *Salmonella* from Africa, finding parallel reduction in biofilm formation between isolates of *S*. Enteritidis and *S*. Typhimurium due to SNPs in the biofilm production master regulator *csgD* or its promoter[434]. They were able to show the phenotype of *csgD* impairment and extrapolate to historic isolates of S. Typhimurium ST313. Given our access to more recent ST313 isolates, some of which also show decreased ciprofloxacin susceptibility (DCS), it would be useful to phenotype a larger set of isolates for their biofilm morphology, follow this by screening of isolates with diverse biofilm morphologies, and look for SNPs shared or divergent between these isolates.

9.2 Effects of ciprofloxacin on *Salmonella* invasion of host cells

With the exception of our pilot study of a *S*. Typhimurium D23580 TraDIS library infection of intestinal organoids, all of our studies were conducted on bacterial cultures. While this was an important first step, we recognize the necessity of studying the response of bacteria

to ciprofloxacin in the context of host cells. While bacteria may encounter antimicrobials in the environment prior to entering a host, the greatest exposure to antimicrobials would be during an infected person or animal that gets treated with antimicrobials. However, the concentration of drug that bacteria are exposed to depends on the drug pharmacokinetics and pharmacodynamics, and interaction of the host immune system. Sakoulas *et al.* demonstrated that clinical isolates *S. enterica* serotype Newport pre-treated overnight with 0.25x MIC of ciprofloxacin showed no difference compared to non-treated bacteria in neutrophil killing assays. However, they found that bacterial survival was significantly reduced if pre-treated with 1x MIC ciprofloxacin and then 0.25x MIC of immune factor cathelicidin LL-37, suggesting a synergistic role for ciprofloxacin and LL-37 in enhanced neutrophil killing of *Salmonella* species causing meningitis[404]. It would be beneficial to perform similar studies using gut-relevant immune cells, such as macrophages, to understand the effect of ciprofloxacin on *Salmonella* invasion and replication. Given our prior assessment of bacterial subpopulations that form under ciprofloxacin treatment, it could be relevant to treat *S*. Typhimurium with ciprofloxacin for two hours, similar to many of our previous assays, and then measure invasion and intracellular replication. Moreover, imaging of the infected cells could help elucidate whether bacterial morphology (elongated versus non-elongated bacteria) influences infection potential.

In addition to studying the dynamics of macrophage infections after *Salmonella* pre-treatment with ciprofloxacin, it would also be useful to investigate the effect of *Salmonella* infection on cells that have been incubated in medium containing ciprofloxacin. Anuforom *et al.* showed that *S*. Typhimurium SL1344 had reduced invasion of J774 murine macrophages when the macrophages were treated with ciprofloxacin, but there was greater adhesion to cells[365]. A study by Tarazona *et al.* demonstrated that *Brucella militensis* infecting canine macrophages exposed to ciprofloxacin had fluoroquinolone MICs ten times higher than bacteria grown in non-exposed macrophages. However, despite the acquisition of GyrA mutations in the ciprofloxacin-exposed set, the mutants did not infect new macrophages at a higher rate than the wild-type bacteria[579]. Barcia-Macay found that intracellular exposure of *Staphylococcus aureus* to ciprofloxacin was bacteriostatic even at the human C_{max} (total drug concentration), in contrast to much higher levels of bacterial killing extracellularly[580]. In contrast, Rajagopalan-Levasseur *et al.* found a 10-fold reduction in intracellular *Legionella pneumophila* bacteria that were grown in macrophages in ciprofloxacin-containing medium compared to those in non-treated medium[581]. This indicates the important role of host cells in modulating drug exposure to bacteria inside of cells and thus the need for further

investigation of how host cells influence drug availability and bacterial intracellular survival. Furthermore, antimicrobials including ciprofloxacin have been noted to have a direct effect on macrophages, including by influencing macrophage gene expression. For instance, Marquez *et al.* demonstrated that treatment of macrophages with ciprofloxacin upregulates the Mrp4 transporter, which may be involved in ciprofloxacin efflux[582,583]. Sanchez *et al.* showed that pre-treatment of gland polymorphonuclear neutrophils, macrophages, and blood monocytes with tumour necrosis factor and then exposure to ciprofloxacin increased killing of intracellular *S. aureus*. However, this synergistic effect only occurred for antimicrobials that can independently target intracellular *S. aureus*[584]. Interestingly, Anuforom found that the combination of ciprofloxacin-treatment and SL1344 infection increased IL-1β and TNF-α expression far more than ciprofloxacin- or bacterial-exposure alone[365]. Infecting *S*. Typhimurium in the presence of ciprofloxacin-exposed macrophages for the purpose of measuring intracellular invasion and replication efficiency, macrophage gene expression, and morphological characteristics of drug-exposed macrophages and bacteria would be a useful set of experiments to further understand bacteria-macrophage-drug interactions. It would be particularly interesting to compare this for isolates with different invasion potentials and antimicrobial susceptibilities to determine what and how large a role macrophages play in limiting infection by invasive and drug-resistant organisms.

9.3 Interaction of *S*. Typhimurium with additional antimicrobials

Finally, we recognize that ciprofloxacin, and fluoroquinolones more broadly, represent only one class of antimicrobials effective against susceptible invasive *Salmonella* infections. While we chose to focus on ciprofloxacin for our studies, it would be worthwhile also exploring the dynamics of *S*. Typhimurium growth in the presence of other antimicrobials and in conjunction with cells. Most pressingly, it would be informative to conduct similar studies on the interaction of bacteria and macrophages with azithromycin, which has gained favour as a widespread prophylactic and reducer of childhood mortality[585–587]. Azithromycin acts on bacteria by inhibiting protein synthesis and is known to readily enter cells, making it an effective antimicrobial for treating intracellular infections[588]. Azithromycin has been recognized to have immunomodulatory effects, including stimulating the downregulation of

CD80 and CD86 as well as suppression of IL-6, IL-10, and TNF-α in LPS-treated dendritic cells and CD4+ cells[589]. In addition, it has been shown to alter the effect of transcription factor NFκB and modulate mucin production, expression of macrophage surface receptors, and autophagy[588]. Given the widespread effects azithromycin has on cells and that it has a mechanism of action on bacteria distinct from fluoroquinolones, it would be useful to study in greater depth. This is particularly true given that our transcriptional studies of S. Typhimurium D23580 treated with azithromycin revealed a distinct set of differentially expressed genes from bacteria treated with ciprofloxacin. This suggests that any transcriptomics or imaging of macrophages treated with azithromycin would behave and look distinct to those treated with ciprofloxacin. A study of co-cultured macrophages and fibroblasts with *Pseudomonas aeruginosa* showed that azithromycin dampens the inflammatory response, and a separate study of cystic fibrosis mice found that azithromycin treatment reduced expression of inflammatory cytokines IL-1β, CCl-2, and TNF-α[590,591]. Given its strong immunomodulatory role and efficacy at bacterial killing, it would be relevant and important to further investigate the effects of azithromycin, particularly in the context of invasive *S*. Typhimurium for which there are limited treatment options in some regions.

As antimicrobial resistance grows, it becomes increasingly important to fully understand the effects of antimicrobial treatment on bacteria and cells. By doing so, it may be possible to discover cases in which certain antimicrobials will not be effective and may lead to greater resistance. Our work has begun to show the widespread effect of ciprofloxacin treatment on *S*. Typhimurium bacteria, and future work will seek to build upon this foundation to better explain bacteria-drug interactions and influence appropriate drug usage.