Colonisation of the gut microbiome by Escherichia coli during international travel

Chapter Five

General Discussion

The increase in levels of AMR bacteria, infections and mortality across the globe is concerning. The risk of an AMR infection poses a danger for one's health, especially in immunocompromised people or those undergoing surgery [137, 138]. An increase in globalisation and international travel is driving the spread of AMR between countries [136, 149], with the gut microbiome acting as a reservoir through which travellers are acquiring and importing AMR bacteria [151]. Perturbations to the gut microbiome are some of the factors most associated with this acquisition [47], but the extent to which the microbiota is involved is unclear. However, if a change to the gut microbiome is associated with an increase in ESBL-E acquisition, then this suggests that the microbiome plays a vital role in creating an effective colonisation resistance to ESBL-E. In this thesis, we explored whether the gut microbiome and individual taxa help prevent the acquisition of ESBL-E.

5.1 How ESBL-E interacts with the gut microbiome as a whole

As the gut microbiome acts as a reservoir for AMR bacteria, the potential for onwards transmission of AMR genes to commensal organisms, opportunistic pathogens or other hosts highlights a concerning problem. In **Chapter 2** we showed how the structure of the microbiome – its bacterial richness and d"iversity – was not correlated to the acquisition of ESBL-E, as it does not significantly differ between travellers acquiring ESBL-E and those remaining uncolonised. It did also not differ amongst the pre-travel microbiomes and travellers who did not go on to acquire ESBL-E did not cluster

separately from those who did, meaning that there is no common microbiome structure that can preclude the invasion of ESBL-E.

Previous studies on the gut microbiome of travellers also found that the microbiome is not correlated with the acquisition of MDR *Enterobacteriaceae* [249, 139, 140], meaning that our results are in concordance with the current literature. However, these studies are limited either by a small sample size [140], or by using 16S rRNA gene sequencing [249] or meta-transcriptomics [139] which have a lack of resolution to species level. These were the main reasons why our WMGS approach on a large data set from longitudinal samples was required. In **Chapter 2**, we also analysed the functional potential of the microbiome and calculated the abundance of genes involved in SCFA production, which are a proxy of good intestinal health [67]. These results were consistent with the microbiome structure analysis as we did not find any groups of metabolic pathways associated with the acquisition of ESBL-E. As we found that ESBL-E can invade the microbiome without causing a significant shift to its structure, it is not unlikely that the metabolic profile of the microbiome also remains stable within our detection limit. The functional composition has been shown to remain relatively stable, even reducing the efficacy of donor microbiome engraftment during faecal microbiome transplantation in recipients with a higher functional redundancy in their gut microbiome [58]. A limitation of the analyses in **Chapter 2** is that we calculated gene abundance, which represents metabolic *potential*; so a follow-up study of transcriptomics or metabolomics analysis could be carried out to determine if there is a higher functional redundancy or specific protective metabolic pathways in travellers who did not acquire ESBL-E than in those who did. A strength of our study, however, was the ability to separate the statistical analysis by geographical region and maintain a sufficiently large sample size, so that the results are applicable per region. Rates of acquisition of ESBL-E can vary quite drastically by country [47], so it was important to analyse at both a global and a regional scale to see if the conclusions are consistent, or if there were regional variations that lead to a different interaction between the gut microbiome and invading ESBL-E, which there were not. Even though travellers will interact with different factors and perturbations that vary by country, the gut microbiome seemingly responds in a similar manner within the scope of our study.

Consistently throughout **Chapter 2**, the onset of travellers' diarrhoea was significantly associated with the acquisition of ESBL-E. Others have already determined that diarrhoea and invasion of MDR bacteria are closely linked [47], for example a reduced species richness in the inflamed gut affecting colonisation resistance [89], or

because *Enterobacteriaceae* are adapted to colonising and thriving in inflammation [94, 95]. Of the traveller microbiome studies, one study found that although the microbiome was not associated with the acquisition of MDR *Enterobacteriaceae*, the pre-travel microbiota did predispose to the onset of travellers' diarrhoea [139]. The analyses in **Chapter 2** could be repeated on pre-travel samples but to instead determine if the onset of travellers' diarrhoea could be predicted. As the onset of travellers' diarrhoea drives an increased acquisition of ESBL-E, better understanding what drives the onset of diarrhoea could then, by proxy, be used to understand the acquisition of ESBL-E. As discussed in **Chapter 2**, ESBL-E is still able to colonise in those without travellers' diarrhoea, but not at the same rate in those with diarrhoea, meaning this is viable future research.

5.2 How ESBL-E interacts with individual species

Given that the microbiome as a whole does not differ between people who do and do not acquire ESBL-E during travel, the question is if individual species or strains have important interactions with ESBL-E. Commensal species are known to contribute to a functioning colonisation resistance to invading pathogens [183, 188, 189], but pathogenic bacteria may utilise their virulence mechanisms to overcome or bypass host defences and colonise the environment [78]. Discovering if there are specific species or a collection of species that ESBL-E are not able to outcompete with could be exploited in the development of probiotics for travellers.

In **Chapter 2** we investigated the taxa that, longitudinally, were significantly associated with the acquisition of ESBL-E. The results show that members of *Bacteroides* and *Citrobacter* had an increase in prevalence and/or abundance in travellers who remain negative for ESBL-E colonisation, suggesting that these species may be protective against ESBL-E. *Bacteroides* species are known to generally be metabolically beneficial for the intestinal health [187, 188, 189], and in a study comparing the gut microbiome of ESBL-E carriers vs non-carriers in Thailand, *Bacteroides uniformis* was found to be positively associated with ESBL-E non-carriers [186], supporting our conclusion that it may be protective. However, an intestinal inflammatory host response, as inducible by ESBL-E, can decrease abundance of *Bacteroides* [94]. Therefore, *Bacteroides* may appear protective in those not colonised with ESBL-E and not experiencing a host immune response, simply as its abundance in

those invaded by ESBL-E was decreased. We show that for *B. cellulosilyticus* and *B. ovatus* there is both an increase in abundance in travellers remaining negative for ESBL-E as well as a decrease in abundance in those who acquired ESBL-E. Together, these suggest that *Bacteroides* may offer protection against ESBL-E but may also have an abundance decrease *in response to* ESBL-E colonisation. This does highlight a limitation of our study, however, as the faecal samples were only collected before travel and immediately upon return, so the chronology of species interactions during travel cannot be determined. To ensure strong conclusions, additional sampling during travel should be taken.

Microcins are known to have strong antibacterial properties and are produced by *Enterobacteriaceae* to be used against other members of the family [141, 83]. It was especially interesting, therefore, that *Citrobacter*, a genus in the family *Enterobacteriaceae*, was also strongly associated with travellers remaining negative for ESBL-E. In **Chapter 3**, we tested the ability of *C. amalonaticus* – the *Citrobacter* species most frequently acquired during international travel to Laos [155] – to compete with commensal and ESBL-producing *E. coli in vitro*. *C. amalonaticus* has been shown to outcompete *C. rodentium*, a model bacterium used in mice to closely represent human enteropathogenic *E. coli* infections in humans, in a mouse infection experiment [143], and is therefore a promising organism to research. Although in **Chapter 2** the *Citrobacter* species of interest was identified as *C. freundii*, the taxonomic identification was limited as *C. freundii* is more present in reference databases as it is more frequently the cause of clinical infections. Our method of taxonomic identification was therefore bias by more present species, so equal numbers of reference genomes should have instead been used to ensure a fairer approach.

We found that *C. amalonaticus* was not able to outcompete either antimicrobial-sensitive or ESBL-producing *E. coli* in nutrient broth *in vitro*. A follow up experiment of growing *E. coli* in the presence of supernatant from *C. amalonaticus* (and other *Citrobacter*) could be performed to confirm the absence of microcins, as this would not be confounded with the effects that *E. coli* may have on the growth of *C. amalonaticus*. There are many metabolic process and host-bacterial interactions that occur *in vivo*, for example the production of immunomodulatory molecules like SCFAs [67], tryptophan [71] or secondary bile acids [69], that were not considered in the *in vitro* experiment. Considering these points, we conclude that *C. amalonaticus* does not directly inhibit the growth of *E. coli* through the use of targeted molecules like microcins, but as there were many metabolic process left unexplored there is potential

for further experiments. An interesting result to note, however, is that one of the *E. coli* isolates from phylogroup A was by far the least able to outcompete *C. amalonaticus*. Discussed further below, members of phylogroup A were the most frequently acquired *E. coli* strains during international travel. If *in vivo* the strains of *Citrobacter* are able to compete with *E. coli*, this may explain how *Citrobacter* is positively associated with travellers who remain uncolonised by ESBL-E in **Chapter 2** as the acquired strains of *E. coli* are the easiest to outcompete.

5.3 How ESBL-E interacts with commensal *E. coli*

It was interesting to note that in **Chapter 2**, we did not detect a significant change in relative abundance of *E. coli* during travel, regardless of ESBL-E acquisition, suggesting that invading *E. coli* have the ability to displace commensal *E. coli*. A study on the metagenomes from 22 independent cohorts of FMT recipients shows a large amount of variability in strain displacement, and that this occurs in a strain-specific manner [250]. Many species, typically the common commensals, frequently showed a donor-recipient strain coexistence, whereas other species showed either a donor or recipient dominance with less frequent coexistence. The outcomes of *E. coli* were not summarised due to a lack in statistical power, but this supports the idea of invading *E. coli* interacting with resident *E. coli*. Strain displacement is a promising avenue for decolonisation of ESBL-E as a preliminary study on the effect of FMT on ESBL-E found a reduction in its abundance and the total amount of ESBL genes [251]. Although the current literature has a strong focus on entire gut microbiome replacement, research into how individual species may interact is still warranted.

In **Chapter 4**, we describe the commensal population of *E. coli* and show how there is a clear temporal change to the detected strains. The commensal population consisted of predominantly phylogroups A and B2 which, even though phylogroup B2 is most frequently detected in extra-intestinal infections [101, 102], was consistent with the limited literature that focused on commensal populations of *E. coli* in wealthy countries [218, 223, 228]. However, we show that during travel there is a clear temporal shift to the *E. coli* phylogenetic distribution towards phylogroups A and B1. Studies on commensal *E. coli* in low- and middle-income countries (LMICs) are very limited, but a high proportion of phylogroups A and B1 have been found in both Mali and Benin in West Africa [229]. If other LMICs were to contain a similar proportion of phylogroups,

it could explain the shift we detected in the travellers. Our results indicate that the invading *E. coli* frequently displace commensal *E. coli* but not in a discernible way between MDR and non-MDR *E. coli*.

Invading *E. coli* employ virulence mechanisms to overcome resident bacteria and host defences in order to colonise [78]. In **Chapter 4**, we investigated whether invading ESBL-producing *E. coli* possessed more virulence genes that potentially contribute to their displacement of commensal *E. coli*. Interestingly, we found that post-travel *E. coli* had significantly fewer virulence genes than pre-travel *E. coli*, independently of ESBL-E acquisition. In a study tracking commensal *E. coli* in a Parisian population between 1980 and 2010, an increase in virulence potential was attributed to both an increase in STs of phylogroup B2, which typically contains more virulence genes [223], and in virulence gene frequency independently of ST frequency [228]. We determined that this significant reduction in virulence is attributed to the temporal loss of phylogroups B2 and D as these contained significantly more virulence genes than phylogroups A and B1. The interpretability is limited, however, by the virulence factor database containing bias towards annotating genes that are required for long term colonisation as virulence genes, which, when calculating gene count, are indistinguishable from genes actually involved in strain-strain competition. Only 10 travellers used antibiotics during travel, so there is no selecting pressure for *E. coli* harbouring AMR genes that could explain the strain displacement. As it is seemingly not dependent on total number of virulence genes, further analyses should be carried out to determine what traits invading ESBL-E possess that allow them to displace resident strains, for example pangenomic analysis to identify genes that cluster strongly with invading *E. coli* and not in the strains that were displaced.

5.4 Future perspectives

Presented in this thesis is an implementation of WMGS in the study of the gut microbiome and its role in the acquisition of AMR during international travel. Despite the foundation of this thesis being a study with one of the most comprehensive traveller gut microbiome data sets, there are still many unanswered questions surrounding microbiome data analysis.

With the increased accessibility to generating sequencing data, traveller study designs must better capture how dynamic a microbiome can be on a day to day basis. Sampling during travel ensures that we do not disregard transient microorganisms, especially as these still have the capability to transmit their AMR genetic material to the resident members of the gut microbiome. Additionally, colonisation resistance to invading pathogens may not always come in the form of total exclusion, but rather a speedy decolonisation triggered by the commensal microbiome, and without consistent sampling these relevant interactions may be missed. In this thesis, the acquisition of ESBL-E is strongly associated with the onset of travellers' diarrhoea, but further sampling during travel could clarify the order in which this occurs. Understanding how frequently ESBL-E elicits an immune response in the healthy intestinal tract, or simply takes advantage of the already inflamed environment could, for example, be used to alter guidance towards traveller behaviour or discover if some gut microbiomes are more resilient to diarrhoea and in turn protected against ESBL-E colonisation.

The future of metagenomics research is bright, especially with the introduction of new, accessible sequencing technologies like long-read sequencing. Long-read sequencing allows for an easier construction of genomes from a metagenomic sample, which can help increase the resolution to which microbiomes are typically analysed. Our findings in chapter 4 exemplify a high level of variation within a single species and that these strains correlate with travel, yet current metagenomics research, due to technical limitations, typically predict the quantity of the entire species. Combining short-read and long-read sequencing to produce high quality genomes of individual strains will shape the future of microbiome research by improving taxonomic identification and better recognising dynamics like strain replacement. A challenge for future research, however, is to keep up the momentum in software for accurate bioinformatics analysis. Statistically analysing microbiome data is notoriously complicated, so new tools must consider the inherent complexity.

Comprehensive microbiome research needs to expand further than metagenomics alone. The complexity of a microbiome is more than just the presence of microorganisms, which is why efforts to integrate metagenomics, transcriptomics and metabolomics are pivotal to the field. Standardising the processes in which these data are produced would harmonise research across institutes, allowing for comparability and meta-analyses. The sheer complexity of the gut microbiome means that discovering a species' role in an environment is not easily replicated in the laboratory, but a multi-omics approach may pinpoint genes of interest and metabolic pathways that lead

to the observed phenotype. The interplay between a single species and the entire gut microbiome means that discovering its role may, in practice, be difficult to replicate due to the exponential knock on effects, but a multi-omics approach would better understand its central role and allow researchers to correctly manipulate the gut microbiome to protect against invasion from pathogenic bacteria.

As the world moves to a more connected and interdependent place, the rates at which international travellers contribute to the spread of AMR will continually rise. We must urgently minimise the global transmission of AMR in order to ensure the clinical use of current antibiotics. The research in this thesis highlights a potential avenue in which to achieve this – colonisation resistance against the acquisition of ESBL-E. We have shown that this is not clearly achievable via the gut microbiome in its entirety, rather individual species that are similar to the invading species. As the technologies involved in microbiome research improve, new targets for interventions to alter one's susceptibility to invading pathogens may be discovered. However, due to the sheer complexity of the gut microbiome, the amount of sampling must be scaled up to incorporate how dynamic the host-bacteria and bacteria-bacteria interactions are on a day by day basis.