# Use of lineage specific bacteriophage to counter antibiotic resistant *Escherichia coli* sequence type 131 (ST131)

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**Date of submission:** 31st March 2022

**Word Count:** 31,583

**Number of figures, tables and text boxes:** 14

**Declaration:** Submitted by Jessica Helen Forsyth, to the University of Exeter as a thesis for the degree of Masters by Research in Biological Science, March 2022.

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# Abstract

The emergence and rapid dissemination of multidrug resistant pathogens presents a huge and growing global concern. *Escherichia coli* (*E. coli*) sequence type (ST) 131, has been recognised as a major *E. coli* lineage responsible for the spread of multidrug resistance, and is a common cause of life-threatening infections in clinical settings. In recent years there has been increasing interest in the use of bacteriophages in the treatment of MDR infections. The use of bacteriophages with some breadth of host range can be desirable so that licensed products are able to target a range of clinical infections. Using a unique method of quantifying host range, we provide evidence that the type of environment sampled from, and the host strain used for isolation are both significant determinants of the host range of phages isolated. Using competition experiments we also provide evidence that by combining the selective pressure of phage predation with that imposed by the presence of a probiotic competitor, significant reductions in the fitness of the MDR pathogen ST131, can be achieved. Although fitness trade-offs resulting from bacteriophage resistance are predicted by life-history evolution, we demonstrate that resistant mutants do not pay a cost for resistance but instead appear to benefit from it with the relative fitness of resistant mutants greater than their susceptible counterparts. Overall, this study draws attention to the value of the environment as a source of naturally occurring antimicrobials, provides direction for future sampling attempts to promote the isolation of phage with the greatest therapeutic potential and demonstrates how the efficacy of such phage can be enhanced through their synergistic application with probiotic strains such as *E. coli* Nissle 1917. This study and those that follow will provide invaluable contributions to the ever-

27 growing body of phage research that will ultimately form part of the solution in the

28 fight against AMR.

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29 # General Introduction
30

31 The discovery of antibiotics revolutionised the field of medicine and resulted
32 in significant reductions in human morbidity and mortality (Lee et al., 2013).
33 Common infections became easier to treat and routine medical procedures safer
34 to conduct. However, the widespread dependency on antibiotics which ensued
35 from their success generated a strong selective pressure favouring the evolution
36 of resistant bacteria. As antibiotic use and overuse has continued, the selection
37 for resistance has strengthened and it has become increasingly apparent that the
38 evolutionary arms race between resistant bacteria and antibiotics, is one that is
39 being lost (Ragheb et al., 2019). Not only does resistance reduce the efficacy of
40 already prescribed antibiotics, it also creates a barrier to the development of new
41 ones, as it is unprofitable to invest in a product that will soon be ineffective (Gould
42 and Bal, 2013). It is inevitable that with increasing resistance will come increasing
43 fatalities from those infections that are no longer responsive to antibiotic
44 treatment. Indeed, the Interagency Coordination Group on Antimicrobial
45 Resistance (IACG) predicts that the 700,000 deaths globally per year caused by
46 drug-resistant diseases could rise to 10 million by 2050 (IACG, 2019). This is not
47 just a problem for public health but for all aspects of economic and social
48 development and therefore requires action on a global scale (Jasovsky, et al.,
49 2016).

50 Multi-drug resistant (MDR) bacteria are regarded as the most critical players
51 in the antibiotic crisis. Broadly speaking, MDR bacteria are defined as those that
52 are resistant to more than one antimicrobial agent (Magiorakos et al., 2012). This
53 increased resistance diminishes the number of antibiotics available for effective
54 treatment, leaving infections caused by MDR bacteria challenging to treat. It is

important to note that even when bacteria remain responsive to select antibiotics, the delay that resistance causes in administering the appropriate drug can often be fatal (Pitout and Laupland, 2008). In the most extreme cases, some highly resistant, gram-negative, bacteria have acquired resistance to nearly all available antibiotics (Breijyeh, Jubeh and Karaman, 2020; Magiorakos, et al., 2012). It is essential that any efforts to develop new antimicrobials, or alternative antimicrobial treatments, are concentrated on these MDR pathogens. Steps towards this prioritisation were made in 2017 when the World Health Organisation (WHO) published a list of antibiotic resistant priority pathogens (WHO, 2017). Members of the most critical group, that is those posing the greatest threat to human health, were made up of MDR bacteria including various Enterobacteriaceae such as *Klebsiella* and *E. coli.* Reported to be the most common gram-negative pathogen in humans, the inclusion of *E. coli* in this critical group is of considerable concern (Rasheed, et al., 2014).

*E. coli* is a normal constituent of the intestinal microflora of humans and other warm-blooded animals (Kaper, Nataro and Mobley, 2004; Köhler and Dobrindt, 2011). While many strains are harmless, some can cause serious infections (Kaper, Nataro and Mobley, 2004). Such strains are referred to as pathogenic *E. coli* and are grouped based on the site at which they cause infection. Intestinal pathogenic *E. coli* (IPEC) are obligate pathogens and are responsible for infections within the gut (Köhler and Dobrindt, 2011). Extra-intestinal pathogenic *E. coli* (ExPEC), although able to asymptomatically colonise the gastrointestinal tract for prolonged periods of time, will only cause infection on gaining access to niches outside of the gut, such as the urinary tract or bloodstream (Köhler and Dobrindt, 2011). Infections caused by ExPEC, including urinary tract infections (UTIs) and bacteraemia, are becoming increasingly challenging to treat owing to

81 the emergence of resistance to first line and last-resort antibiotics such as
82 fluroquinolones and third-generation cephalosporins (Pitout, 2012). With
83 epidemiological surveys reporting nearly 60% of UTI ExPEC isolates as resistant
84 to three or more antibiotic classes, the importance of efforts to control the
85 continued rise and spread of MDR in *E. coli* is clear (McNally, et al., 2019;
86 Loddenkemper, Sagebiel and Brendel, 2002). To do so, we require an
87 understanding of the resistance determinants responsible (Xu et al., 2018).

88 One of the most important mechanisms of resistance in *E. coli* is the
89 production of Extended-Spectrum-β-Lactamases (ESBLs) (Liu, et al., 2016).
90 ESBLs are enzymes capable of hydrolysing and conferring resistance to
91 commonly used β-lactam antibiotics, such as penicillins and cephalosporins
92 (Paterson and Bonomo, 2005; Shaikh, et al., 2015). Most ESBL genes are
93 located on mobile genetic elements known as plasmids (Bonnet, 2004). Often,
94 these plasmids co-harbour resistance genes for multiple antimicrobial classes
95 meaning the acquisition of such a plasmid can easily give rise to MDR in the host
96 (Rawat and Nair, 2010; Mitra et al., 2019). Through horizontal gene transfer, this
97 MDR can readily be disseminated among other bacterial strains and even
98 between species (Sun et al., 2019). Currently, the most common ESBL enzyme
99 worldwide is the plasmid-encoded CTX-M type which are defined by their
100 enhanced hydrolytic activity against third-generation cephalosporin antibiotics
101 such as cefotaxime (Bonnet, 2004). The most common ESBL gene among *E. coli*
102 isolates is $bla_{CTX\text{-}M\text{-}15}$ (Rawat and Nair, 2010; Mohsin et al., 2017). The
103 widespread emergence of the $bla_{CTX\text{-}M\text{-}15}$ gene is particularly concerning due to
104 its association with the highly successful *E. coli* clone sequence type 131 (ST131)
105 (Isgren et al., 2019).

The population structure of *E. coli* is characterised by the presence of four main phylogenetic groups, named A, B1, B2 and D (Herzer et al., 1990). Typically, commensal strains of *E. coli* belong to phylogroups A and B1 whilst those strains responsible for extra-intestinal infections are associated with phylogroups B2 and D (Charkraborty et al., 2015; Mosquito et al., 2015; Nowrouzian et al., 2009). Within these groups, distinct lineages are defined using multi-locus-sequence-typing (MLST), a powerful phylogenetic approach that analyses the internal fragments of seven housekeeping genes to determine a strains sequence type (ST) based on the alleles present at each locus (Pitout and Finn, 2020; Nicolas-Chanoine, Bertrand and Madec, 2014). ExPEC strains are the leading cause of human extraintestinal infections globally, yet only a small subset of ExPEC lineages are to blame for the vast majority of infections (Manges et al., 2019). One such lineage is sequence type (ST) 131 whose evolution has been marked by a series of clonal expansions that has resulted in the emergence of three major and divergent clades, A, B and C (Price et al., 2013). It is the expansion of clade C that is of particular interest due to its association with MDR. Clade C, otherwise known as ST131-H30, is composed of strains that contain allele 30 of the type 1 fimbriae adhesin gene fimH (Olesen et al., 2014). The H30 clade is further subdivided into two sister clades, H30R and H30Rx, that are distinguished by their alternative antimicrobial resistance profiles (Kondratyeva, Salmon-Divon and Navon-Venezia, 2020). Subclone H30R was established through the acquisition of several mutations within the quinolone resistance determining regions of *subunit A of DNA gyrase* (gyrA) and *subunit A of DNA topoisomerase* (parC) which supplied strains with chromosomally encoded resistance to fluroquinolones (Cummins et al., 2021). It is thought that the discovery and subsequent licensing of fluroquinolones in the 70's and 80's for the treatment of

extraintestinal infections, such as UTIs, likely contributed to the emergence of this fluroquinolone resistance subclone (Stoesser et al., 2016; Emmerson and Jones, 2003). The second sister clade of H30, named H30Rx, emerged shortly after and is characterised by the carriage of the ESBL encoding $bla_{CTX\text{-}M\text{-}15}$ gene (Mamani et al., 2019). Across several studies, including Merino et al. (2016), almost all isolates that produce CTX-M-15 cluster in this subclade, whose disproportionate association with sepsis has been documented and is of considerable concern (Olesen et al., 2014; Price et al., 2013). It is important to recognise that the high prevalence of $bla_{CTX\text{-}M\text{-}15}$ among ST131 isolates associated with extraintestinal infections is not a result of the widespread acquisition of resistance across the lineage but due to the expansion of this highly successful subclone (Olesen et al., 2014). As such, the temporal increase in resistance that has been observed amongst ST131 has a strong clonal basis and a great deal of research has explored the mechanisms behind the success of ST131 (Olesen et al., 2014).

The success of ST131 can be attributed to a unique combination of characteristics. As the defining characteristic of H30Rx strains, MDR is thought to contribute greatly to the success of ST131 yet there is considerable evidence throughout the literature that undermines the role of MDR in the widespread proliferation of ST131. Firstly, *E. coli* clones, such as ST405, ST648 and ST410, in possession of similar virulence, but more impressive resistance profiles, have not experienced the same global dissemination and success (Pitout and Finn, 2020). Additionally, although the emergence of ST131 led to the displacement of ST405, a lineage with a reportedly higher rate of MDR, it had little effect on established clones such as ST73 that is largely dominated by antibiotic susceptible isolates (Matsumara et al., 2013; Pitout and Finn, 2020). This suggests that although the resistance profile of H30Rx subclone isolates is what

158 has brought the ST131 lineage to the forefront of global attention, it does not
159 appear to be exclusively responsible for its success (Kallonen et al., 2017). An
160 enhanced level of virulence has also been proposed as a driving factor behind
161 the epidemic rise of the ST131 lineage which appears to be supported by the
162 disproportionate association between the H30Rx subclone and sepsis (Merino et
163 al., 2016). Although ST131 strains do indeed possess a high number of virulence
164 associated genes (VAGs), including *ferric aerobactin receptor gene (iutA), ferric*
165 *yersinibactic uptake receptor gene (fyuA)* and *enterobacterial complement*
166 *resistance protein (traT),* this does not differ significantly from that of other ExPEC
167 lineages (Riley, 2014). It is likely that although ST131 does not possess
168 significant differences in its total virulence, it possesses a specific set of virulence
169 factors that result in success. This idea is supported by the identification of a
170 virotype scheme whereby virotypes are defined by the presence or absence of
171 particular virulence genes (Blanco et al., 2013). Interestingly in the same way
172 strains cluster into distinct ST131 clades, there appear to be distinctive
173 associations with certain virotypes, with H30Rx strains largely belonging to
174 virotype A. (Jamborova et al., 2018). In murine models of sepsis, isolates
175 belonging to virotype A were shown to possess significantly higher levels of
176 virulence than isolates belonging to virotype D in which most non-H30 ST131
177 strains are clustered (Mora et al., 2014). This suggests that the possession of a
178 unique combination of virulence-associated genes (VAGs), or specific virotype,
179 has contributed to the epidemic expansion of the H30Rx subclade (Olesen et al.,
180 2014). A third factor believed to have contributed to the lineages' success is the
181 carriage of low cost, AMR encoding plasmids (Pitout and DeVinney, 2017). Most
182 ST131 strains harbour plasmids that encode the $bla_{CTX\text{-}M\text{-}15}$ gene responsible for
183 ESBL production (Nicolas-Chanoine, Bertrand and Madec, 2014). Complete

184 genome sequencing of EC958, the most studied strain of ST131, revealed the
185 appearance of a 135.6 kb plasmid responsible for harbouring 12 antibiotic
186 resistance genes (Schembri et al., 2015). It is not just the possession of this
187 plasmid that is significant, but the low cost at which it is carried. Typically, plasmid
188 carriage involves significant fitness costs to the host, but ST131 appears to
189 ameliorate this through the possession of multiple compensatory mutations
190 (Pitout and DeVinney, 2017). Such mutations act to stabilise plasmid carriage
191 and means the acquisition of MDR for ST131 has been relatively cost free.
192 Although the individual characteristics of the ST131 lineage are not unique and
193 are shared by other ExPEC lineages, it is the alliance of these traits in a single
194 lineage that is responsible for its success. Efforts to understand the factors
195 contributing to the successful proliferation of the H30Rx subclone will be essential
196 to develop targeted intervention measures and the prevention of the emergence
197 of other pathogenic clones with such epidemic potential (Price et al., 2013).
198 Although the dominance of ST131 in the problem of AMR is concerning, its
199 targeted removal may provide us with the opportunity to achieve a significant
200 reduction in the prevalence of MDR ExPEC infections worldwide. The MDR
201 profile of ST131 eliminates conventional antibiotics as a method to achieve this,
202 and therefore development of alternative antimicrobial strategies is urgently
203 needed. One strategy that has gained renewed interest in recent decades, is the
204 use of bacteriophages.

205 Bacteriophages (phages) are naturally occurring viruses that infect and kill
206 bacteria (Principi, Silvestri and Esposito, 2019). They were first discovered in the
207 early 20th century by Félix d'Hérelle who proposed their potential use in the
208 treatment of bacterial infections (d'Hérelle, 1917; Lin, Koskella and Lin, 2017).
209 However, with the discovery of convenient, broad-spectrum antibiotics in the

post-war era, the therapeutic potential of phages became largely overlooked by Western medicine (Summers, 2012). As these have begun to fail, there has been a renewed interest in the use of phages as a complement, or alternative, to antibiotics in the treatment of MDR infections (Lin, Koskella and Lin, 2017). There are three main features of phages that make them particularly promising candidates as alternative antimicrobials. First is their relatively high specificity of infection, second is their ubiquity in the environment (Hyman, 2019) and third is their ability, as living entities, to counteract the evolution of bacterial resistance (Sousa and Rocha, 2019).

Bacteriophages are collectively referred to as narrow spectrum antimicrobials due to their high specificity of infection (Nikolich and Filippov, 2020). Many phages are only capable of infecting bacteria belonging to a single species, or even subgroup of that species, although some can target a wider range of hosts (Clokie et al., 2011). The narrow spectrum activity of phages was once considered to be a barrier to their application in mainstream medicine, as it necessitates more accurate diagnosis of the causative agent of an infection (Hill, Mills and Ross, 2018; Melander, Zurawski and Melander, 2018). However, it is now beginning to be regarded as one of their most valued characteristics due to its potential to combat two of the most important issues related to the use of antibiotics, that is, collateral damage to the gut microbiome and the evolution of resistance (Hill, Mills and Ross, 2018). When broad spectrum antibiotics are administered, they not only target the causative agent of the infection but simultaneously eliminate commensal bacteria that constitute the hosts microbiome (Ramirez et al., 2020). In contrast, phage do not indiscriminately kill bacteria and will only target the strain of interest allowing this collateral damage to be largely avoided (Principi, Silvestri and Esposito, 2019). The need to prevent

this damage has become increasingly important as research establishes connections between disturbance of the gut microbiota and chronic illnesses, including cardiovascular and respiratory disease (Zhang and Chen, 2019). In addition, phage specificity means that exposure to the selective pressure that gives rise to resistance will be limited to the targeted bacterial strain. This is in comparison to antibiotic treatment where commensal bacteria, unrelated to the infection, are also exposed to selective pressure. This additional exposure can result in the evolution of resistance in commensal bacteria that then persist and act as reservoirs of resistance for many years to come (Melander, Zurawski and Melander, 2018).

Although the emergence of resistance remains a challenge with phage therapy, the inherent evolvability of phages enables them to counter this resistance by co-evolving with their hosts (Borin et al., 2021). This process of reciprocal adaptation is commonly referred to as antagonistic coevolution, which is defined as 'the reciprocal evolution of host resistance and parasite infectivity' (Borin et al., 2021; Thompson, 1994; Buckling and Rainey, 2002). Although antibiotics are substances active in the treatment and prevention of bacterial infections, they are not living biological entities and cannot coevolve. Emerging resistance is challenged via the production of new antibiotics, but with the evolution of antibiotic resistance rapidly outpacing this, antibiotics fail to offer a sustainable solution to the global resistance crisis. Phages, however, hold more promise of long-term success owing to their ability to nullify bacterial defence mechanisms, such as CRISPR-Cas immunity, through the evolution of counter defence mechanisms, such as anti-CRISPRs (Streicher, 2021; Yu and Marchisio, 2020). In the case of lytic, or virulent, bacteriophages where release of viral particles requires lysis of the host cell, participation in these coevolutionary arms

262 races is essential to their sustained coexistence with rapidly evolving bacterial
263 hosts (Buckling and Rainey, 2002).

264 Despite the capacity of phages to counter mechanisms of bacterial resistance,
265 the existence of asymmetry in these coevolutionary interactions threatens their
266 therapeutic potential (Buckling and Rainey 2002). Numerous studies have
267 reported that while phages readily demonstrate reciprocal evolution of infectivity
268 in response to the evolution of host resistance, bacteria often gain the upper hand
269 in the coevolutionary arms race by evolving resistance that phage cannot
270 overcome (Burmeister et al., 2021). Their inability to keep pace with their hosts
271 is potentially linked to the comparatively small genomes of phages as well as their
272 requirement to attach to bacterial hosts via specific cell surface receptors
273 (Buckling and Rainey, 2002; Stone et al., 2019). Such specificity and reduced
274 evolutionary potential leaves phage with a diminished capacity to adapt to specific
275 host changes, such as the deletion or inactivation of phage receptors observed
276 in some *E. coli* isolates (Bucking and Rainey, 2002; Burmeister et al., 2021).
277 Fortunately, it is possible to buffer against this by employing a range of
278 techniques that act to enhance the adaptability of phages or inhibit the
279 development of phage resistance and subsequently leverage their ability to
280 counter the evolution of host resistance (Monferrer and Domingo-Calap, 2019;
281 Borin, et al., 2021). Such techniques include the use of phage cocktails, which
282 refers to the simultaneous use of multiple phages in a single preparation (Chan,
283 Abedon and Loc-Carrillo, 2013).

284 The theory behind the use of phage cocktails is that the mutations or
285 adaptations that provide a host with phage resistance are often highly specific.
286 By using multiple phages that infect via different mechanisms it is possible to
287 reduce the likelihood of phage-resistant mutants arising as this would necessitate

288 the simultaneous evolution of multiple resistance mechanisms (Oechslin, 2018).
289 The rationale behind the use of phage cocktails can be likened to that of
290 combination therapy used for the treatment of HIV, tuberculosis, and cancer
291 where several drugs that work by different mechanisms are taken together in an
292 attempt to limit the emergence of resistant mutants (Maenza and Flexner, 1998;
293 Johnson, 1994). Additionally, the evolution of resistance often imposes
294 pleiotropic fitness costs (Mangalea and Duerlop, 2020). This may increase host
295 susceptibility to other representatives of the phage cocktail as well as to
296 antibiotics, and may result in the attenuation of virulence, as exhibited by ST131
297 in a murine infection model (Salazar et al., 2021). An important consideration for
298 the design of phage cocktails is the potential for the evolution of cross-resistance
299 where resistance to one phage concurrently confers resistance to other
300 components of the cocktail (Wright et al., 2019). Ensuring the members of a
301 cocktail use different host receptors for infection can be a way to mitigate against
302 this (Nikolich and Filippov 2020; Wright et al., 2018). As well as improving the
303 efficacy and long-term durability of phage cocktails, there are other techniques
304 whose potential should be explored. These include phage training which
305 repeatedly coevolves phages with their target hosts to provide them with a head
306 start in the coevolutionary process by priming them to deal with the host defences
307 that ancestral hosts will undoubtedly evolve (Borin et al., 2021).

308 Phages are not the only microorganisms that possess potential for therapeutic
309 use, with increasing interest in the application of probiotics for the prevention and
310 treatment of infections (Behnsen et al., 2013). ‘Probiotics are defined as living
311 bacteria that, when administered in adequate amounts, confer a health benefit on
312 the host’ (Hotel and Cordoba, 2001). Such health benefits may be achieved
313 through the stimulation of host immunity or via the competitive exclusion of

pathogenic isolates (Mathipa and Thantsha, 2017). First described by Gause in the early 20th century, the competitive exclusion principle states that two populations cannot inhabit the same ecological niche as one will inevitably out-compete the other for limited resources such as nutrients and space (Gause, 1934; Urban, 2009; Paquette et al., 2018). Such competition is faced by members of the diverse microbial community that inhabit the niche of the gastrointestinal tract, including both commensal and pathogenic strains of *E. coli*. It has been suggested that employing probiotic strains that occupy the same ecological niches as those filled by MDR strains would provide the opportunity to exploit ecological competition to eradicate multi-drug strains from the gastrointestinal tract (Tannock et al., 2011; Ljungquist et al., 2020). Such usage of probiotics is referred to as eradication therapy or intestinal decolonisation and is an example of an alternative antimicrobial strategy that can be likened to phage therapy by its classification as 'microbe-based' and 'pathogen-specific' (Hwang et al., 2017). One of the most studied probiotic strains is *E. coli* Nissle 1917, also known by its commercial name of 'Mutaflor' (Behnsen et al., 2013).

*E. coli* Nissle is a probiotic strain that has the potential to be used as a therapeutic intervention for the treatment and prevention of *E. coli* ST131 infections (Pradhan and Weiss, 2020). In 1917, an army surgeon known as Alfred Nissle isolated *E. coli* from the faeces of a German soldier who remained healthy during an outbreak of Shigella that resulted in diarrhoeal disease for many of his comrades (Behnsen et al., 2013). Nissle believed that the soldiers' resistance was a result of his carriage of a strain of *E. coli* that provided protection by inhibiting the growth of enteropathogens, a hypothesis later confirmed by laboratory tests (Sonnenborn, 2016). Sequencing of this isolate, named after Nissle himself, has revealed several fitness factors that can help to explain its

340 successful intestinal colonisation and enhanced competitive ability (Jacobi and
341 Malfertheiner, 2011; Pradhan and Weiss, 2020). These factors most notably
342 include the possession of multiple, and specialised, iron uptake systems
343 (Behnsen et al., 2013) and the production of small peptides known as microcins
344 that possess antimicrobial properties (Patzer et al., 2003). Nissle has had some
345 success as a therapeutic agent and is commercially available as single and mixed
346 probiotic supplement in several countries in Europe (Sonnenborn, 2016).

347 Through the intestinal decolonisation of ST131, the occurrence and spread of
348 clinically significant infections, such as bacteraemia and UTIs, may be reduced.
349 As an ExPEC strain, ST131 can innocuously colonise the intestinal niche and
350 persist as a commensal component of the gut microbiota for extended periods of
351 time (Sarkar et al., 2018). However, once outside of this niche, these facultative
352 pathogens act opportunistically, resulting in a wide range of extraintestinal
353 infections. Indeed, it has been recognised that the most important risk factor for
354 the development of bloodstream infections, of which *E. coli* is the leading cause,
355 is previous colonisation with ESBL-producing bacteria, such as ST131 (Vila et
356 al., 2016; Kohler et al., 2022). Using probiotics to reduce, and potentially
357 eliminate, the asymptomatic carriage of such bacteria would allow us to reduce
358 the chance of such infections arising. For example, the removal of ST131 from
359 the guts of patients would reduce the risk of faecal shedding of the pathogen and
360 the subsequent UTIs that result. Additionally, reduced intestinal carriage would
361 also prove beneficial in disrupting human-to-human oral-faecal transmission of
362 ST131, which is thought to be the most frequent route of transmission for 'human-
363 adapted ESBL-*E. coli*' (Day et al., 2019). Although the intestinal decolonisation
364 of pathogens using probiotics has been achieved, such successes are often
365 preceded by the administration of antibiotics (Tannock et al., 2011). In these

366 cases, the probiotic capitalises on the discriminative action of antibiotics which
367 frees up space in the intestinal niche. This apparent reliance on antibiotics for
368 probiotic success, suggests that the collapse of antibiotic effectiveness may also
369 decrease the therapeutic value of probiotics.

370 The concurrent application of phage and probiotics may present a powerful
371 and novel interventional strategy for the eradication of MDR *E. coli* from the
372 gastrointestinal tract (Sarkar et al., 2018). Singularly, phage therapy and
373 probiotics are promising therapeutic agents, but both have recognised
374 drawbacks. Our unique strategy aims to combine the selection pressure imposed
375 by phage predation with that of ecological competition that stems from the
376 introduction of a probiotic, to lower the frequencies of MDR strains in the gut. The
377 benefits of this two-tiered approach are achieved via the positive interactions that
378 exist between phage and probiotic. Although the evolution of phage resistance is
379 almost inevitable, it is also likely to result in pleiotropic fitness trade-offs that may
380 have a negative impact on bacterial colonisation, growth, persistence, virulence
381 and recognition by the host immune system (Sausset et al., 2020; Mangalea and
382 Duerkop, 2020; Smith, Huggins and Shaw, 1987). Such trade-offs will enhance
383 the capacity of the probiotic strain to effectively outcompete it and improve the
384 efficacy of this strategy. Additionally, previous, unpublished, experiments have
385 identified frequency dependent competition between Nissle and ST131, as would
386 be predicted by classical competition theory (Barron, 2018; Levin, 1988). As a
387 result, a reduction in the frequency of the pathogenic strain through phage
388 predation provides the probiotic with an improved ability to outcompete its
389 pathogenic competitor. Such a strategy that capitalises on the evolution of
390 resistance as opposed to being immobilised by it is undoubtedly attractive, but its

391 implementation will rely heavily on the experimental confirmation of both the
392 fitness costs and frequency dependent interactions that it is dependent upon.

393 The present study was designed to investigate the potential for the use of
394 lineage-specific bacteriophages in the targeted decolonisation of MDR *E. coli*
395 ST131. This has involved the identification of environments in which ST131-
396 specific phages could be isolated from, as well as subsequent analysis of the
397 comparative effectiveness of such environments, and isolation hosts, on the
398 acquisition of phages most desirable for therapeutic application. The
399 characteristic of host range was used to measure this and was quantified using
400 a unique method that utilises a modified version of the Shannon diversity index
401 to provide a more comprehensive classification of phage host range (Shannon,
402 1948). The characterisation of a subset of isolated phages using next generation
403 sequencing has also been carried out to provide an insight into the diversity of
404 ST131-specific phages present in both natural and artificial sewage
405 environments. In addition to the focus on the environmental isolation of phage,
406 the study set out to investigate a new two-tiered strategy that combines the use
407 of phages and probiotics. The aim of such a strategy is to provide a therapeutic
408 technique capable of buffering against the evolution of phage resistance and
409 subsequently increasing the success of decolonisation attempts. We explore the
410 effectiveness of phage and probiotics, in isolation and in combination, on the
411 ability to drive down frequencies of ST131. We also assessed the impact of phage
412 cocktails on the evolution of resistance and its associated fitness costs. As
413 antibiotics continue to lose their effectiveness, research such as this into the
414 potential of alternative antimicrobial strategies will be vital if we are to retain the
415 ability to control and manage the public health threat posed by MDR pathogens
416 such as *E. coli* ST131.

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# Chapter one

## The environmental isolation of bacteriophages infective against MDR *E. coli* ST131

### Introduction

The rise and spread of antibiotic resistance is one of the greatest global threats to health, food security, and societal and economic development of the 21st century (WHO, 2020; Sabtu, Enoch and Brown, 2015). With high rates of resistance to frequently used antibiotics now observed world-wide, common and life threatening bacterial infections, such as urinary tract infections (UTIs) and sepsis, are becoming increasingly untreatable (WHO, 2020). Accompanying this worrying rise in resistance is the scaling down, and in some case abandonment, of antibiotic discovery research and development programmes (Hutchings, Truman and Wilkinson, 2019). With the threat of a return to a pre-antibiotic era creeping ever closer, the need to invest in the development of alternative antimicrobials has never been greater. One such alternative is phage therapy, which utilises naturally occurring bacterial viruses, known as bacteriophages, to treat bacterial infections (Lin, Koskella and Lin, 2017). Previously side-lined by the Western world in favour of broad-spectrum antibiotics, the practise has gained renewed interest in recent years with an increasing number of cases demonstrating the successful application of phages in clinical settings. One such example is the effective use of a three-phage cocktail to treat a 15-year-old girl suffering with a disseminated drug-resistant *Mycobacterium abscessus* (Dedrick et al., 2019). Such successes, along with the testimony provided by their

443 sustained and commonplace use in other parts of the world, serve as promising
444 indications of the therapeutic potential of bacteriophages for the treatment of
445 clinical bacterial infections (Nale and Clokie, 2021).

446 Gram-negative (GN) bacteria pose a substantial threat to public health due to
447 their high resistance to antibiotics combined with their capacity to cause serious
448 disease (Oliveira and Reygaert, 2021). The protective outer membrane and
449 expression of efflux pumps characteristic of this group, contributes to the intrinsic
450 resistance associated with GN pathogens such as Enterobacteriaceae, *P.*
451 *aeruginosa* and *A. baumannii* (Galindo-Mendez, 2020; Exner et al., 2017). One
452 GN pathogen class of undeniable significance in the antibiotic crisis is
453 extraintestinal pathogenic *E. coli* (ExPEC) (Galindo-Mendez, 2020). Responsible
454 for a range of community-onset and hospital-acquired infections, the high and
455 rising rates of resistance observed among ExPEC, particularly to fluroquinolones
456 and third-generation cephalosporins, is of considerable and growing concern
457 (Gregova and Kmet, 2020). A disproportionate number of antibiotic resistant
458 strains of ExPEC *E. coli* belong to a genotype of *E. coli* referred to as sequence
459 type (ST) 131 (Pitout and DeVinney, 2017). The dissemination of this lineage is
460 of particular concern due to its carriage of $bla_{CTX\text{-}M\text{-}15}$, a gene that confers
461 resistance to β-lactam antibiotics via the production of extended-spectrum β-
462 lactamases (ESBLs). The production of ESBLs represent the main mechanism
463 of resistance to β-lactams in *E. coli* strains with $bla_{CTX\text{-}M\text{-}15}$ the most widely
464 distributed gene encoding ESBLs globally (Louka et al., 2020; Zhang et al., 2014).

465 Antimicrobial control strategies must focus on clonal groups that pose the
466 most urgent threat to public health (CDC, 2019). Amongst *E. coli*, the high
467 epidemic potential of the ST131 lineage is widely recognised, with some

468 academics arguing that its enhanced virulence, intestinal colonisation, and
469 transmission capability may even warrant its classification as a hyperendemic
470 clone (Olesen et al., 2013; Dautzenberg et al., 2016; Bernasconi et al., 2020).
471 The dominance of such epidemiologically successful clones in the antibiotic crisis
472 is troubling, and demonstrates the vital importance of targeted intervention
473 measures, such as lineage-specific bacteriophages, in preventing the spread of
474 MDR (Dautzenberg et al., 2016).

475 The marked contribution of ST131 to the increasing prevalence of MDR,
476 alongside its association with intestinal colonisation, renders it an ideal candidate
477 for the targeted use of bacteriophages (Dautzenberg et al., 2016). That is, the
478 employment of lytic phages to infect and kill populations of pathogenic bacteria
479 (Loc-Carrillo and Abedon, 2011). Central to this concept is the high host
480 specificity of phages, with many only able to infect a subset of strains of a single
481 bacterial species (Loc-Carrillo and Abedon, 2011). Identifying and exploiting
482 phages that directly, and exclusively, target ST131, would allow the intestinal
483 decolonisation of this pathogen to be achieved without causing widespread
484 disruption to the commensal constituents of the gut microbiome. This would be
485 invaluable progress considering the adverse effects that often ensue from the
486 indiscriminate mode of action of broad-spectrum antimicrobials (Zhang and
487 Chen, 2019). However, in order to exploit the therapeutic potential of ST131-
488 specific phages, we must first investigate how best to isolate, and subsequently
489 characterise, these natural antimicrobial agents (Kim et al., 2019).

490 Despite the extensive research conducted in nations such as Georgia, Russia
491 and Poland, there are still many obstacles to the clinical application of phages in
492 the Western world (Gorski et al., 2018). One such obstacle is that the findings of
493 many of the studies conducted have not been made readily available to the

494 international scientific community with many published in Russian, or journals
495 inaccessible in the West (Sulakvelidze, Alavidze and Morris, 2001; Chanishvili et
496 al., 2001). In addition to this, those studies whose results can be accessed often
497 fail to meet the rigorous safety standards required for modern clinical trials.
498 Ultimately, this means that whilst we continue to advance the field of phage
499 science through the investigation of outstanding research gaps, we must also set
500 out to re-learn and strengthen knowledge that has already been acquired.

501 Although it is known that phage specific to ST131 are present in the
502 environment, our understanding of the most efficient way to isolate them remains
503 poor. Phages are believed to be the most abundant microorganisms on earth with
504 at least one type of phage capable of infecting every strain of bacteria (Clokie et
505 al., 2011; Keen, 2016). As obligate pathogens, they are present in the
506 environment in coexistence with their bacterial hosts (Batinovic et al., 2019). As
507 a gut pathogen, *E. coli* ST131 and the phages that target it, can be readily isolated
508 from a range of sewage environments, including wastewater facilities and animal
509 sludge (Anastasi et al., 2012). Although the literature provides a broad indication
510 of where to look, it fails to address how isolation attempts may be optimised to
511 ensure the most successful capture of ST131-targeting bacteriophages, or other
512 specific lineages, or indeed how to isolate phage enriched for particular
513 characteristics. In this context, success is not only indicated by the abundance of
514 target phage but also by the usefulness of those isolated for clinical application.
515 It is therefore also necessary to find ways to tailor isolation methods towards the
516 preferential isolation of phages that possess the most promising therapeutic
517 potential.

518 One measure of therapeutic potential is bacteriophage host range. This refers
519 to the diversity of hosts a phage can infect and is a property amongst which

520 phages exhibit substantial variation (de Jonge et al., 2019). A consequence of
521 such variation is that phages differ in their effectiveness as phage therapy
522 candidates, with broad host range phage generally favoured due to their potential
523 to be effective against a wider range of bacterial isolates (Brussow, 2019). The
524 population structure of ST131, characterised by the clonal expansion of related
525 lineages emphasises the value of optimising phage host range for therapeutic
526 application. Having undergone approximately 40-50 years of intense microbial
527 evolution, significant diversification has taken place amongst the ST131 lineage
528 with some suggesting that the lineage can no longer be regarded as 'a unified
529 entity' but as a group of distinct clonal subgroups (Olesen et al., 2014). Population
530 genetics analysis of ST131 isolates has revealed that the rapid spread and
531 dissemination of ST131, and the MDR now associated with it, can largely be
532 attributed to the emergence of the H30R and H30Rx subclones (Price et al.,
533 2013). This is further confirmed by the fact that most of the ST131 isolates that
534 produce CTX-M-15 belong to the H30Rx subclone (Nicolas-Chanoine, Bertrand
535 and Madec, 2014). The phylogenetic clustering of strains that are responsible for
536 the majority of MDR infections can be used to our advantage by focussing
537 attempts to achieve the targeted removal of problematic MDR strains on these
538 specific clonal subsets (Stoesser et al., 2016). These subsets are themselves
539 comprised of several strains which necessitates the use of broad host range
540 phage that are capable of infecting as many possible genotypes of this subgroup
541 as possible. For the purposes of this study, host range is defined as the number
542 of ST131 strains a phage can infect and its level of infectivity (measured by
543 PFU/mL) on these strains. In line with our aim of isolating ST131-specific
544 bacteriophages, it would be beneficial to isolate phage capable of infecting as
545 many genotypes in the ST131 clonal complex as possible without causing

disruption to the commensal *E. coli* strains that co-colonise the gut. Therefore, although broad host range is desired at the strain level, a narrower host range is preferred at the species level. Despite the clear predilection for broad host range phage, the majority of standard isolation protocols fail to select for any particular host range (Hyman, 2019).

In this study we modified the Shannon Weaver diversity index to develop a novel method for scoring host range. Using this modified index, we set out to explore factors that may enhance the success of attempts to isolate ST131-specific phages, with a specific preference for those with broader host ranges. Traditional measures of host range only recognise the span of hosts that a phage can infect through the use of binary presence/absence data and fail to acknowledge variation commonly seen in the level of infectivity (PFU/mL) amongst these hosts (Holtzman et al., 2020). Here we replace this approach with a method that assigns each phage with a single host range value that accounts for both the number of host strains it infects (host richness) as well as the level of infectivity on these strains (host evenness). Such an approach will contribute to achieving more robust and informative biological conclusions than those obtained using former measures of phage host range.

In this chapter we explore the effect of sampling environment and isolation host on the success of isolation of ST131-specific phages. We set out to test the effectiveness of isolation from four distinct sewage environments; clinical sewage, agricultural waste, community wastewater and activated sludge, each chosen based on previous evidence of successful coliphage isolation. Considering its classification as a MDR strain and the widespread use of antibiotics in hospital settings, we hypothesised that clinical sewage would be the most successful sampling environment for the isolation of ST131 targeting phage.

572 In addition, most protocols use a single host strain for the isolation of
573 bacteriophages from environmental samples, yet the use of a single host is likely
574 to limit the types of phages that can be isolated, especially in terms of their host
575 range (Hyman, 2019). We therefore investigated whether the strain used for
576 isolation of bacteriophages had a significant impact on their host range. To do so
577 we used nine unique strains of *E. coli* ST131, confirmed by PCR and sequence
578 analysis, and hypothesised that isolation host would indeed be a significant
579 predictor of phage host range (Anne Leonard and colleagues, unpublished). The
580 ability to identify any associations between sampling environment and isolation
581 host on the success of isolation of ST131 specific phage, would provide valuable
582 direction for future sampling attempts. This, combined with our efforts to
583 characterise a subset of isolated phage via whole genome sequencing, will
584 contribute towards the development of a systematic sampling framework that will
585 improve the ability to obtain phage possessing the most promising therapeutic
586 potential from the environment.

587 # Materials and Methods
588

589

590 ## Environmental samples
591

592 To isolate host-specific phages, materials that were anticipated to contain high
593 levels of *E. coli* ST131 were used as source samples. This included wastewater,
594 activated sludge (product of a biological wastewater treatment method), pig
595 faeces and biogas slurry (by-product of biogas production generated from
596 anaerobic digestion of animal waste and crop residue) as well as hospital sewage
597 (Peces et al., 2022; Xu et al., 2021). Samples were collected in the U.K. between
598 October 2019 and January 2020. Approximately 1 liter of material was collected
599 at each sample location and transported to the lab for immediate processing
600 where it was, where necessary, blended and strained, and subsequently mixed
601 50:50 with 75% glycerol. Samples were then stored at -80$^{\circ C}$.

602 **Table 1.** Information on source samples

| Source category | Sample location | Date of collection | Type of material |
|---|---|---|---|
| Wastewater | Falmouth sewage treatment plant (STP) | 18.10.2019 | Influent wastewater |
| | Ponsanooth sewage treatment plant (STP) | 7.11.2019 | Influent wastewater |
| | Carnon Downs sewage treatment plant (STP) | 7.11.2019 | Influent wastewater |
| Agricultural | Healeys Cyder Farm | 24.10.2019 | Pig faeces (from two animals) |

| | | | |
|---|---|---|---|
| | Ixora Energy limited plant Gorst Energy, Exeter | 30.10.2019 Collected by colleagues at the University of Exeter | Slurry |
| | Fraddon Biogas, St Columb | 1.11.2019 Collected by colleagues at the University of Exeter | Slurry |
| Activated sludge material | Hayle sewage treatment works | 30.10.2019 Collected by colleagues at the University of Exeter | Settle sludge |
| | Falmouth sewage treatment plant (STP) | 17.01.2020 | Activated sludge |
| | Marsh MILLS (SWW) Plymouth | 08.11.2019 Collected by colleagues at the University of Exeter | Settle sludge |
| Clinical waste | Royal Cornwall Hospital (Treliske) | 23.10.2019 | Sewage chamber material |

603

604 **ST131 isolates**

605

606 The *E. coli* ST131 isolates used in this study are listed below. The *E. coli* ST131
607 EC958 reference strain was provided by Matthew Upton of the University of
608 Plymouth and was originally sourced from a patient in the UK in 2005 presenting
609 with a urinary tract infection (Forde et al., 2014). All other ST131 isolates were

610 provided by Anne Leonard of the University of Exeter and were originally isolated
611 from human subjects that had participated in 'The Beach Bum Survey' in 2015
612 (Leonard et al., 2018). Isolates were stored at -80℃ and streaked onto LB agar
613 plates. When grown, single colonies were launched in 6ml of lysogeny broth (LB),
614 and cultures were grown overnight at 37℃ in a shaking incubator (180rpm).

615 Isolates of *Escherichia coli* ST131 used in the study:

616 - EC958
617 - C869E1
618 - C233E3
619 - 343E1
620 - 153C2
621 - C467E1
622 - 302E1
623 - C744E1
624 - C233E1

625

626 ## Isolation of bacteriophages

627

628 *E. coli* ST131-specific phages were isolated from source samples via the
629 enrichment procedure detailed as follows. 1ml of each sample was added to 5ml
630 of sterile LB with the addition of 60µl of *E. coli* ST131. For each sample three
631 replicate cultures were prepared for all nine ST131 isolates and grown overnight
632 at 37℃ in a shaking incubator (180rpm). To remove bacterial debris from the
633 enriched cultures two methods of purification were carried out: filtration and
634 chloroforming. Both methods were used to account for the possibility that some
635 phages may be inactivated by chloroform treatment (Hyman, 2019).

636 • Filtration

637 15ml Falcons containing 2ml of enriched culture material were centrifuged
638 for 20 minutes at 3500rpm. The supernatant was passed through a
639 0.22μm filter and stored at 4$^{\circ C}$.

640

641 • Chloroforming

642 300μl of chloroform was added to 3ml of enriched culture in 15ml Falcons
643 and thoroughly mixed by vortexing. Samples were then centrifuged for 25
644 minutes at 3500rpm and then the supernatant removed and stored at 4$^{\circ C}$.

645

646 Using the conventional double-layer agar method, spot assays were carried out
647 to test for the presence of phage (Sambrook and Russell, 2001). For each
648 sample, 5μl of 10-fold serial dilutions were spotted onto lawns of LB soft agar
649 (0.5%) for each of our 9 ST131 isolates and incubated overnight at 37$^{\circ C}$. The
650 presence of phage was detected in the form of clear plaques and phage titers
651 (PFU/mL) were calculated using the following equation (Baer and Kehn-Hall,
652 2014);

653

654 $$PFU/mL = \frac{Average\ number\ of\ plaques}{Dilution\ \times volume\ of\ diluted\ virus\ added}$$

655

656 **Single plaque purification**

657

658 To ensure isolation of single phage genotypes, the double-layer agar method was
659 used to obtain single phage plaques. To this end, 50μl of each phage sample
660 was mixed with 100μl of the *E. coli* ST131 isolate that the phage was initially

isolated using, and 5ml of LB-soft agar (0.5%). This was poured onto the surface of a petri dish containing a layer of solidified LB agar and incubated at 37℃ overnight.

Single plaque morphologies were isolated by picking a single plaque with a sterile pipette tip. This tip was left to stand in an Eppendorf containing 300µl of M9 buffer for 1-2 hours before being removed and the contents of the Eppendorf vortexed. These single-plaque cultures were then filtered or chloroformed into new Eppendorf's and stored at -4℃. If multiple plaque morphologies kept appearing, single-plaque purification was repeated.

## Phage amplification and stock titration

Purified phage was amplified by mixing 6ml of LB medium, 60µl of the host bacterial strain and 200µl of filtered or chloroformed phage and grown overnight at 37℃ overnight in a shaking incubator (180rpm). The contents of glass microcosms were transferred to 15ml Falcon tubes and centrifuged at 3500rpm for 40 minutes. The supernatant was removed, filtered, and chloroformed before being stored at -4℃. Glycerol stocks of these phage (30%) were prepared and stored at -80℃.

Amplified phage stocks were then used to conduct further spot assays using the double layer agar method to assess the infectivity of phage and titrate the stocks.

682 ## Host range determination
683

684 Spot assays using the double-layer agar method were used to determine the host
685 range of amplified phages. 5µl of 10-fold serial dilutions of phage stocks were
686 plated on LB soft-agar (0.5%) lawns of all 9 *E. coli* ST131 isolates and incubated
687 overnight at 37℃. The presence of plaque formation indicated infective capability
688 of the phage against the bacterial isolate used.

689

690 ## Abundance of bacteria in raw samples
691

692 Glycerol stocks of all raw samples were defrosted and vortexed to homogenise
693 any solid debris. 5µl of 10-fold serial dilutions in 0.85% saline solution were
694 spotted in triplicate on ChromoSelect agar (Merck, Darmstadt) and ChromoSelect
695 agar with cefotaxime (8mg/mL) and nalidixic acid (30mg/mL) for selective
696 enrichment of *E. coli* ST131, which is resistant to both these antibiotics. On
697 ChromoSelect agar, presumptive *E. coli* colonies should turn up as blue/dark
698 purple, 'other' coliforms pink and other gram-negative bacteria white.

699

700 ## Phage abundance in raw samples
701

702 Glycerol stocks of all raw samples were defrosted. 5ml of each sample was
703 aliquoted into 15ml falcon tubes and chloroform was added at a ratio of 1:10. All
704 chloroformed samples were centrifuged at 4℃, 3500 rpm for 25 minutes.
705 Supernatant was removed and passed through a 0.22µm filter into a sterile falcon
706 tube. 5µl of 10-fold serial dilutions of phage stocks were plated in triplicate on LB

707 soft-agar (0.5%) lawns of all 9 *E. coli* ST131 isolates and incubated overnight at
708 37℃.

709

## 710 Polyethylene glycol precipitation (PEG) of amplified phage
711

712 In order to obtain enough amplified phage to use in the process of PEG
713 precipitation, we conducted two rounds of phage amplification using 1-liter
714 conical flasks. For each round 240ml LB medium and 250µl of $MgCl_2$ and $CaCl_2$
715 was added to each flask and autoclaved before being inoculated with 2.4ml of
716 the relevant ST131 host and 8ml of the corresponding phage stock. Flasks were
717 incubated in a shaking incubator overnight at 180 rpm at 37℃. Flasks were
718 removed from the incubator and the contents aliquoted into 50ml Falcon tubes
719 each containing 40ml of amplified stock. Chloroform was added to each falcon at
720 a ratio of 1:10 and tubes were inverted several times before being centrifuged for
721 40 minutes at 4℃, 3500 rpm. Supernatant was removed using serological pipettes
722 and decanted into fresh falcon tubes. These were centrifuged again for another
723 40 minutes to pellet any remaining bacterial debris and the supernatant aliquoted
724 off and filtered through a 0.22µm filter. These amplified phage stocks were then
725 titrated by plating 10-fold serial dilutions on 0.5% soft agar plates containing the
726 relevant ST131 isolate.

727 PEG precipitation was carried out to concentrate the amplified phage material
728 into a smaller volume. A 24% (w/v) PEG 8000, 1.5M NaCl solution was made up
729 and autoclaved before being stored at 4℃. Two 250ml centrifuge bottles
730 (Beckman Coulter) and one 50ml Falcon were set up per phage containing a 2:1
731 ratio of phage to PEG solution and were mixed well by shaking before leaving
732 overnight at 4℃.

250ml bottles were centrifuged at 9,000 x g, 4°C for 1 hour (Beckman Coulter rotor JLA 16.250) and the Falcon tubes were centrifuged at 6,000 x g, 4°C for 1 hour (rotor JS 5.3). Supernatant was decanted into a sterile Duran bottle leaving approximately 10ml residual volume behind. A serological pipette was used to wash down the sides of the bottle and pipette up and down to resuspend any pelleted phage. The bottles were swirled around before all residual volume was decanted into one Falcon tube per phage. A small aliquot (50/60µl) of this residual volume was removed to measure phage concentration prior to chloroforming. Residual volume was passed through a 0.22µm filter using 2ml of M9 buffer to wash through at the end. Chloroform was added at a ratio of 1:1 with the phage material and centrifuged for 30 minutes at 3500 rpm at 4°C. Supernatant was removed, filtered (0.22µm) and stored at 4°C. Titrations were conducted for the residual volume prior to chloroforming, the chloroformed stock ready for DNA extraction and the supernatant aliquoted from the initial PEG precipitation spins. DNA extraction only proceeded too if titer of chloroformed stock was at least $10^9$/ml in concentration.

## Phage DNA extraction

100kDa 15ml Amicon filters (Merck, Darmstadt) were used to concentrate the chloroformed and syringe filtered PEG precipitated phage stock. Filters were centrifuged at 3500 rpm, 18°C for 15 minutes. 400µl of DNase I buffer (diluted 10x in M9) was added to the filter and pipetted up and down to wash the lysate. After this samples were centrifuged for 10 minutes (3500 rpm, 15 min) and the supernatant discarded. Subsequently, 200µl of DNase I buffer in M9 was added

758 to the filter and pipetted up and down gently 5 times to resuspend the phage from
759 the filter.

760 10 units of DNase I were added to the Amicon filter and pipetted up and down to
761 mix before incubating at room temperature for 15 minutes. Ensuring there is no
762 supernatant at the bottom of the filter, 5ml of DNA extraction buffer was added
763 and tube inverted several times to ensure the DNase I is not retained in the
764 residual volume. The Amicon filter was then centrifuged at 18$^{\circ C}$ for 10 minutes at
765 3500 rpm and the supernatant then discarded.

766 775µl of DNA extraction buffer was added to the filter and pipetted up and down
767 before transferring the total volume remaining in the filter (~975 µl) into a 5ml
768 Eppendorf. To take the final volume in the Eppendorf to ~1ml, 25µl of 50mM
769 EDTA was added as an additional method of inhibiting the action of DNAse I. The
770 Eppendorf was placed in a water bath pre-heated to 75$^{\circ C}$ for 5 minutes in order
771 to inactivate DNAse I.

772 Norgen Biotek Phage DNA Isolation Kit was used to extract DNA as per the
773 manufacturer's instructions. Samples were analysed using Qubit and Nanodrop
774 and their quality was checked by running on a 0.8% agarose gel before being
775 stored at -80$^{\circ C}$.

776

## 777 Calculation of host range and host susceptibility

778

779 Two methods were used to calculate phage host range.

780 1- Percent (%) infection measure – host range was calculated as a
781 percentage using the following equation;

782

783 $$\frac{Number\ of\ ST131\ hosts\ infected}{Total\ number\ of\ ST131\ hosts\ screened\ against\ (9)} \text{ x } 100$$

784

785 2- Diversity measure – host range was compressed into a measure of
786 diversity. To calculate the Shannon diversity index (H) for each phage, the
787 following equation was used; (Shannon and Wiener, 1948).

788

789 $$H = -\sum_{i=1}^{S} p_i * \ln p_i$$

790

791 $p$i = n/N where n is the number of individuals of a given type/species and N is the
792 total number of individuals in a community.

793 For each phage, PFU/mL on each host (n) was divided by the total PFU/mL
794 across all 9 hosts (N) used for host range testing. In this way host genotypes
795 were treated as species and phage plaques as the number of individuals in a
796 community.

797

798 Host susceptibility

799 The susceptibility of all 9 ST131 hosts was calculated using the following
800 equation;

801 $$\frac{Number\ of\ phage\ capable\ of\ infecting\ host}{Number\ of\ phages\ isolated\ in\ total\ (116)}$$

802 ## Data Analysis
803

804 The effect of sampling environment and isolation host on bacteriophage host
805 range was analysed using generalised linear modelling, conducted in RStudio
806 1.1.442. Model checking was conducted to assess the homogeneity of variance
807 and normality of residuals which confirmed the use of parametric tests was
808 appropriate. Model simplification was conducted by replacing the four-level factor
809 of environment with a two-level factor of 'All other environments' and 'Community
810 wastewater' due to non-significant values being achieved when all four levels
811 included. Model simplification was also conducted through the grouping of our 9
812 isolation hosts into three groups (A,B,C). In both circumstances, comparison of
813 the model with and without these simplifications were carried out to detect
814 whether the grouping of environments or hosts led to a loss of explanatory power
815 via F tests of change in deviance.

816 # Results

817

818 ## Patterns in phage host range and the value of our diversity-
819 ## based measure of host range

820

821 A total of 116 phages capable of infecting at least 1 of a panel of 9 ST131 isolates
822 were isolated, and their host range characterised. Figure 1 illustrates the
823 patterns of host range identified.

824 All 116 phages can be seen to broadly fall into one of three groups. The first group
825 is inclusive of phages that infect all ST131 isolates with the overall exception of
826 EC958. The second group are those that appear to preferentially infect isolates
827 C233E1, C233E3, C869E1, C744E1 and C467E1 and the third is comprised of
828 phages that almost exclusively infect isolates 343E1, 153C2 and 302E1.

829 These patterns of infectivity indicate that our 9 host isolates can also be
830 categorised into three groups. Our reference ST131 strain EC958 proved to be
831 a very poor isolation host being resistant to the majority of isolated phage. The
832 EC958 isolate alone forms host group A. Host group B is comprised of those
833 hosts primarily infected by group 2 phage (C869E1, C233E3, C467E1, C744E1
834 and C233E1) and group C is made up of host isolates 343E1, 153C2 and 302E1.

835 The infection of group B hosts does not provide resistance to the infection of
836 group C hosts and vice versa. Of the 116 phages isolated, 99 infected at least
837 one group C host. Of this 99, a total of 54.5% exclusively infected hosts belonging
838 to group C. 61 phages infected at least one group B host. Of this 61, a total of
839 26.2% exclusively infected hosts belonging to group B.

840 Panel A and B of Figure 1 demonstrate the advantage of the use of our novel
841 diversity-based measure of host range. Phage characterized with identical host
842 ranges when using a percent infection measure (Figure 1B) are shown to possess
843 differences in their infective capacity when data on the level of infectivity
844 (PFU/mL) is incorporated into the analysis (Figure 1A). This is further supported
845 by Figure 2 which illustrates differences in the infectivity of two phages whose
846 host range is identical when using a percent infection measure of host range.
847 Phage A in Figure 2 would not be a suitable candidate for phage therapy due to
848 its low titer at high dilutions, a detail that would otherwise be overlooked without
849 the inclusion of infectivity data in the characterisation of host range.

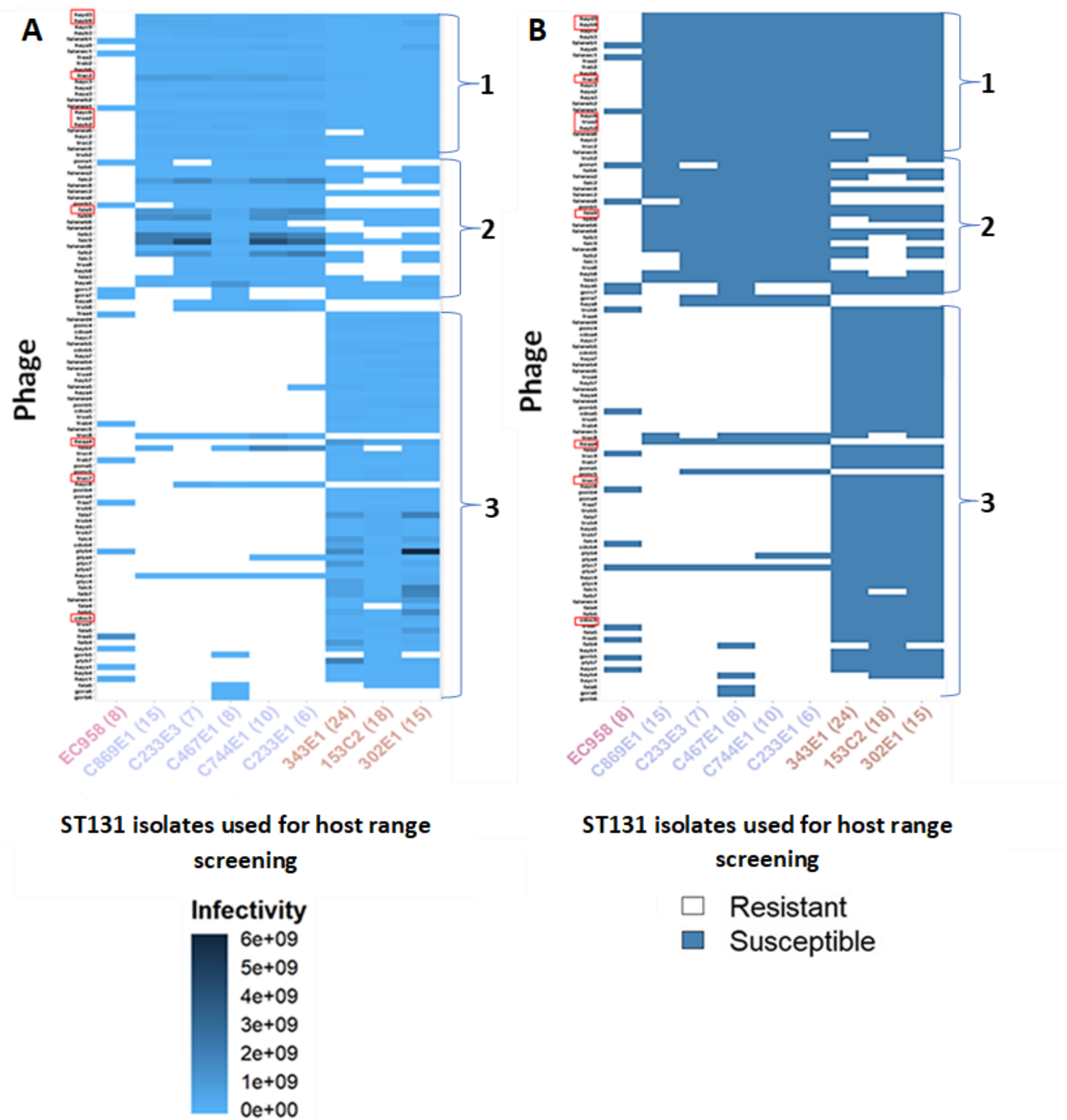


**Figure 1.** Heatmaps demonstrating the infectivity of 116 isolated phages on 9 ST131 isolates. **(A)** Phage infectivity is represented by PFU/mL values (diversity measure). Darker blue represents higher PFU/mL values. **(B)** Phage infectivity is represented using a binary Yes(susceptible)/No (resistant) system (% infection measure). Phages that have the same host range in plot **B** possess distinct and important differences in the evenness of their infectivity across ST131 isolates in plot **A.** The 9 phages highlighted in red are those whose DNA has been sequenced using Illumina next-generation sequencing (NGS) in the order of HayD3, HayB9, FracC2, HayC6, TruA2, HayB2, FalA9, HeaA4, Truc7 and CdoC5. The ST131 isolates on the X-axis have been colour coded according to the three host isolate groupings identified, Group A (EC958), Group B (C869E1, C233E3, C467E1, C744E1, C233E1) and Group C (343E1, 153C2, 302E1). Phages have been broadly classified as belonging to group 1, 2 or 3 based on shared patterns of infectivity.

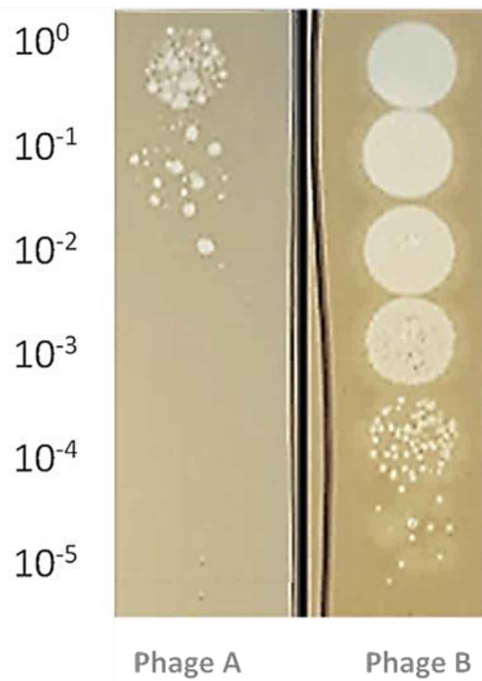


**Figure 2.** Differences in the level of infectivity (PFU/mL) of two phages (A and B) when plated on ST131 isolate C869E1. Image shows the resulting plaques from 5µl of 10-fold serial dilutions (represented on the Y-axis) of phage stocks plated on LB soft-agar (0.5%) lawns of ST131 isolate C869E1. The presence of plaque formation indicates infective capability of each phage against C869E1. Figure 2 shows that although phage A and phage B are capable of infecting ST131 isolate C869E1, their level of infectivity (PFU/mL) differs. Such differences are not embodied in a percent infection measure of host range resulting in the basic characterisation of isolate C89E1 as susceptible to infection by both phages. In contrast our diversity measure of host range accounts for differences in the level of infectivity which provides us with the ability to distinguish between phages more accurately in their suitability as phage therapy candidates.

850 ## Genome sequencing reveals an association between isolation
851 ## host and phage host range

852

853 Illumina sequencing was conducted on 10 bacteriophages with varying host
854 ranges. These phages had been isolated from a range of sewage environments
855 using a variety of ST131 hosts. A summary of the output can be found in Table
856 2.

857 Phages were arranged based on similarities in their illumina sequencing output
858 including contig length, family, estimated characterisation and gene count (Table
859 2). The first three phages in Table 2 all share a high degree of similarity in their
860 sequencing output but have each been isolated from a different sewage
861 environment. The factor that appears to unify these phages is their isolation host.
862 Although not identical, each host belongs to the same host grouping (C) identified
863 in Figure 1. The same pattern can be seen with the remaining seven phage
864 whose hosts all belong to host group B.

865 Phages isolated using group B hosts differ most noticeably from those isolated
866 using group C hosts in their contig length, family, estimated characterization and
867 gene count. Phage isolated using group C hosts appear to have longer contig
868 lengths (166744), higher gene counts (267) and belong to the Myoviridae family
869 of phages. They have also all been estimated as being characterized as
870 *Escherichia coli* phage vB_EcoM_G9062. In contrast, phage isolated using group
871 B hosts possess shorter contig lengths (44277-44603), smaller gene counts (72-
872 76), belong to the Siphoviridae family of phages and are all characterized as
873 *Escherichia* virus Golestan. One phage, labelled as C869E1 Frad C2, produced

874 a poor genome assembly composed of 70 contigs and, as such, is not

875 represented in the analysis.

**Table 2.** Summary of Illumina next generation sequencing (NGS) of 9 bacteriophages isolated using a range of ST131 isolation hosts from multiple isolation environments. Phage ID refers to the ST131 host used for isolation, the environment of isolation and a unique identification code. Phages are grouped based on the isolation host groupings (A, B, C) identified in Figure 1. Phage isolated using hosts belonging to the same host grouping (B or C) share strong similarities in their contig length, gene count, family and estimated characterisation and differ from those isolated using hosts belonging to an alternative group. Phage 343E1_Healeys_A4 produced two contigs that are both represented in the table.

| Group | Phage ID | Isolation environment | Isolation host | Host range (diversity measure) | Contig length (bp) | Family | Estimated characterisation | Gene count |
|---|---|---|---|---|---|---|---|---|
| C | **302E1_Truro_C7** | Clinical | 302E1 | 0.94 | 166744 | Myoviridae | *Escherichia* phage vB_EcoM_G9062 | 267 |
| C | **153C2_Cdo_C5** | Wastewater | 153C2 | 0.58 | 166744 | Myoviridae | *Escherichia* phage vB_EcoM_G9062 | 267 |
| C | **343E1_Healeys_A4** | Agricultural | 343E1 | 0.97 | 166744 | Myoviridae | *Escherichia* phage vB_EcoM_G9062 | 267 |
| C | **343E1_Healeys_A4** | Agricultural | 343E1 | 0.97 | 39493 | Autographiviridae | *Escherichia* virus LM33P1 | 50 |
| B | **C233E1_Hayle_B9** | Sludge | C233E1 | 1.85 | 44603 | Siphoviridae | *Escherichia* virus Golestan | 74 |
| B | **C869E1_Truro_A2** | Clinical | C869E1 | 1.69 | 45181 | Siphoviridae | *Escherichia* virus Golestan | 76 |
| B | **C869E1_Hayle_B2** | Sludge | C869E1 | 1.69 | 45181 | Siphoviridae | *Escherichia* virus Golestan | 76 |
| B | **C233E3_Hayle_C3** | Sludge | C233E3 | 1.76 | 44277 | Siphoviridae | *Escherichia* virus Golestan | 72 |
| B | **C467E1_Hayle_C6** | Sludge | C467E1 | 1.70 | 45181 | Siphoviridae | *Escherichia* virus Golestan | 76 |
| B | **C233E1_Fal_Sludge_A9** | Wastewater | C233E1 | 1.52 | 44603 | Siphoviridae | *Escherichia virus Golestan* | 75 |

876 ## Isolation environment and isolation host are significant
877 ## determinants of phage host range

878

879 The abundance of coliforms did not differ significantly between all four sampling
880 environments (Figure 3A; One-way-ANOVA, $F_{3,25}$ =1.145, P>0.05). Evidence of
881 the presence of ST131 was only detected in activated sludge and community
882 wastewater environments with no significant difference between the two in its
883 abundance (Figure 3B; One-way-ANOVA, $F_{1,6}$ statistic= 0.27, P>0.05).

884 To determine if the isolation host and sampling environment were significant
885 determinants of host range, a generalised linear model was conducted and a
886 post-hoc ANOVA test used to determine the significance of both factors. The
887 ST131 host used for isolation was a significant determinant of the host range of
888 isolated bacteriophages (Figure 3C, GLM, ANOVA, $F_{8,104.}$ = 14.04, P<0.00001).
889 The sampling environment was also a significant determinant, with phages
890 isolated from community wastewater environments possessing a significantly
891 greater host range than those isolated from all other environments as determined
892 by one-way ANOVA (Figure 3C, GLM, ANOVA, $F_{1,103}$= 24.24, P<0.0001). Model
893 simplification was carried out through the grouping of all non-significant
894 environments (agricultural, clinical and activated sludge) in order to more clearly
895 distinguish the effect of the wastewater environment on the host range of phages
896 isolated. It is important to note that the clinical sewage environment was
897 underrepresented in our sampling regime with material collected from one site as
898 opposed to three.

899 To further investigate the significant relationship between host range and
900 isolation host, we plotted the average host range (represented as % infection

901 (Figure 4A) and using our modified diversity index (Figure 4B) on a categorical
902 axis of ST131 isolation hosts. For both measures, a GLM was carried out to
903 detect if the variation among phages in their values of host range could be
904 explained by the host on which they were isolated. For the % infection measure
905 of host range, the host range of phages isolated using group C hosts (343E1 and
906 302E1) were not significantly different from the intercept (153C2) but hosts
907 belonging to group A and B were (GLM, $t_{104}$=10.19, p<0.00001). This pattern was
908 also true when using our modified measure of phage host range (GLM, $t_{104}$=9.86,
909 p<0.00001). This provides support for the clustering of group C hosts
910 independent of those belonging to groups A and B. Further support for this was
911 provided when we produced a simplified, three group model where hosts were
912 classified as members of host group A, B or C. For our percent infection measure
913 and novel diversity measure, the results of our 9-host model were replicated with
914 the host ranges of phages belonging to groups A and B significantly different to
915 those belonging to group C (GLM, $t_{110}$=16.04, p<0.00001; GLM, $t_{110}$=16.83,
916 p<0.00001). In both our % infection and diversity index models, group A was not
917 significantly different from group B (TukeyHSD, Group A-Group B, p adj = 0.12;
918 TukeyHSD, Group A-Group B, p adj = 0.7). This is likely related to the wide
919 amount of variation in the values of host ranges of phages isolated using group
920 A host, EC958 which, as seen in Figure 4A/B overlaps with the host range values
921 of phages isolated using isolation hosts belonging to group B. For both measures
922 of host range, a statistical comparison of the 9 host-model against our simplified
923 3 group model was carried out. There was a significant loss of deviance when
924 using the basic percent infection measure of host range which suggests the
925 existence of a substantial amount of within-group variation (ANOVA,
926 $F_{104,110}$=9.64, P<0.0001). This variation is likely to be caused by the deviance of

927 hosts C467E1 and C744E1 from other group B hosts (Figure 4A). Although the
928 use of our simplified model also resulted in a loss of explanatory power when
929 using our modified measure of phage host range, the significance value was far
930 smaller suggesting the existence of a much smaller level of within group variation
931 and the ability to achieve much more consistent results when using our novel
932 measure of host range (ANOVA, $F_{104,110}=2.84$, $P<0.05$).

933 To determine if the host range of isolated phage was determined by the
934 susceptibility of the isolation host to phage infection, a Pearson's product-
935 moment correlation was conducted between the average host range of phages
936 and the susceptibility of hosts to phage infection. Although a correlation was
937 found when using our percent measure of host range (Figure 5A, t=-2.73, df=7,
938 $P<0.03$), no correlation was found when using our diversity-based measure
939 (Figure5B t=-1.21, df=7, $P>0.05$). Figure 5 demonstrates that when using our
940 richer measure of host range, average host range peaks more clearly at
941 intermediate levels of host susceptibility, and at the very least allows us to
942 discriminate more easily between bacterial isolates, demonstrating the utility of
943 our novel measure in detecting patterns in our dataset.

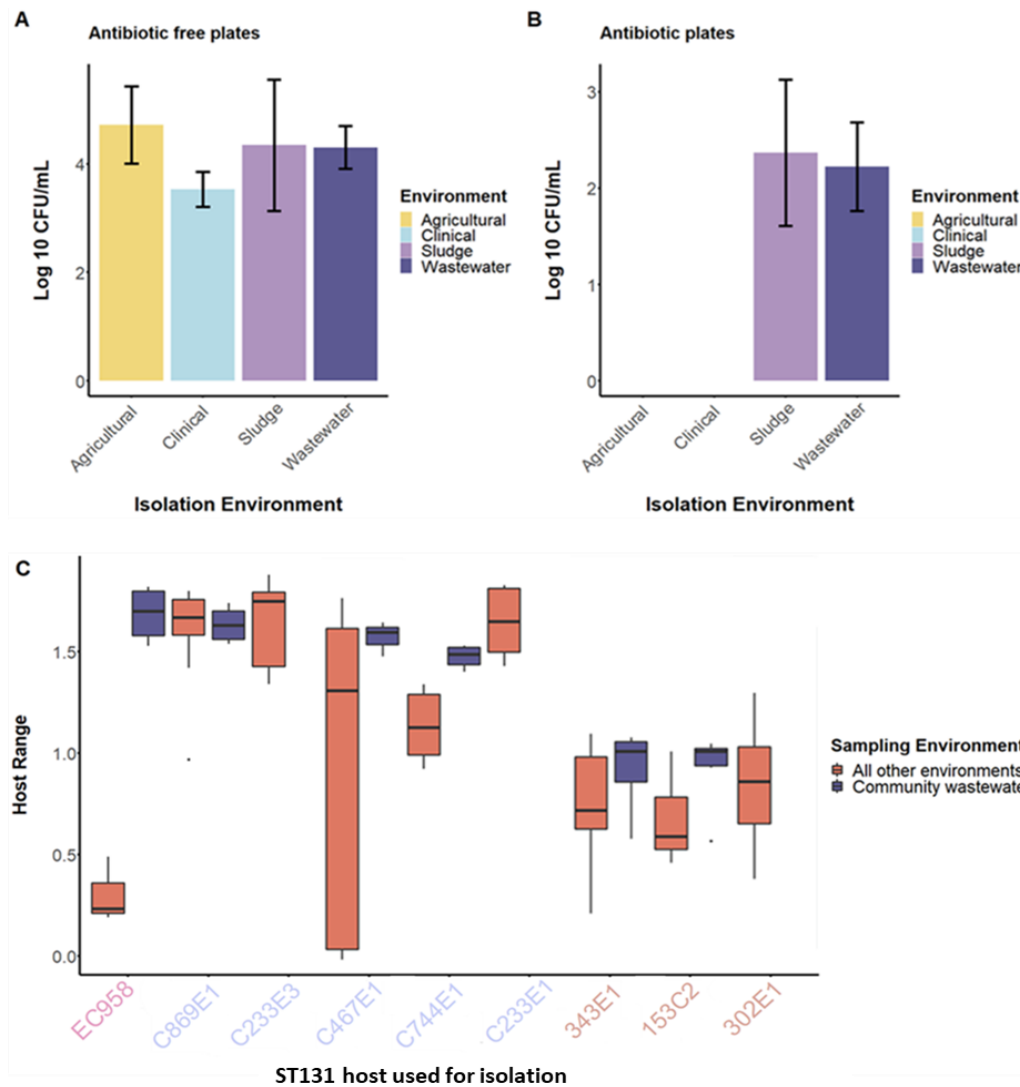


**Figure 3.** Abundance of coliforms in raw environmental samples and the effect of sampling environment and ST131 host on phage host range. **A.** Abundance of total coliforms present in raw environment samples plated on ChromoSelect Agar. Bars represent average CFU/mL (Log10) from three repeats and error bars represent 95% confidence intervals. **B.** Abundance (CFU/mL Log10) of *E. coli* ST131 in raw environmental samples detected by plating on ChromoSelect Agar containing Nalidixic acid (30mg/mL) and Cefotaxime (8mg/mL). **C**. The effect of sampling environment and ST131 host used for phage isolation on the average host range of phages isolated using our novel modified diversity index measure of host range. Four sewage environments were sampled from; agricultural, clinical, community wastewater and activated sludge. The effect of sampling environment and isolation host were both statistically significant. Only the community wastewater environment had a significant effect on host range, so all other environments were grouped for model simplification. Isolation hosts are colour coded according to the host groupings (A, B and C) identified in Figure 1.

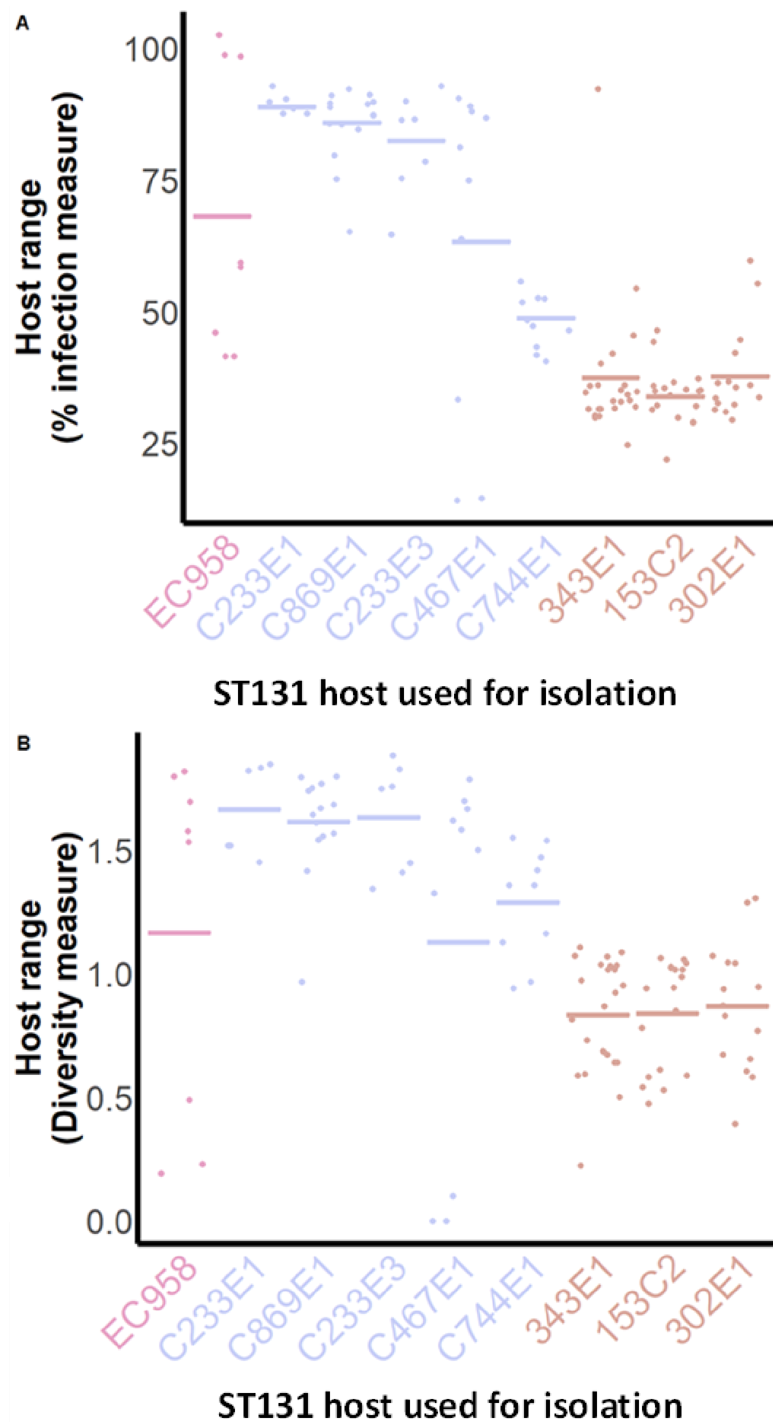


**Figure 4.** The host range of phages isolated on each of the 9 *E. coli* ST131 host strains used in this study. Horizontal lines represent the average host range of phage isolated using each of the 9 hosts. Jittered data points are plotted to demonstrate the variation in host range among phages isolated using the same host. **A** uses the percent infection measure of host range and **B** uses our novel diversity measure of host range. Isolation hosts on the X-axis are colored based on host groupings (A, B and C) identified in Figure 1.

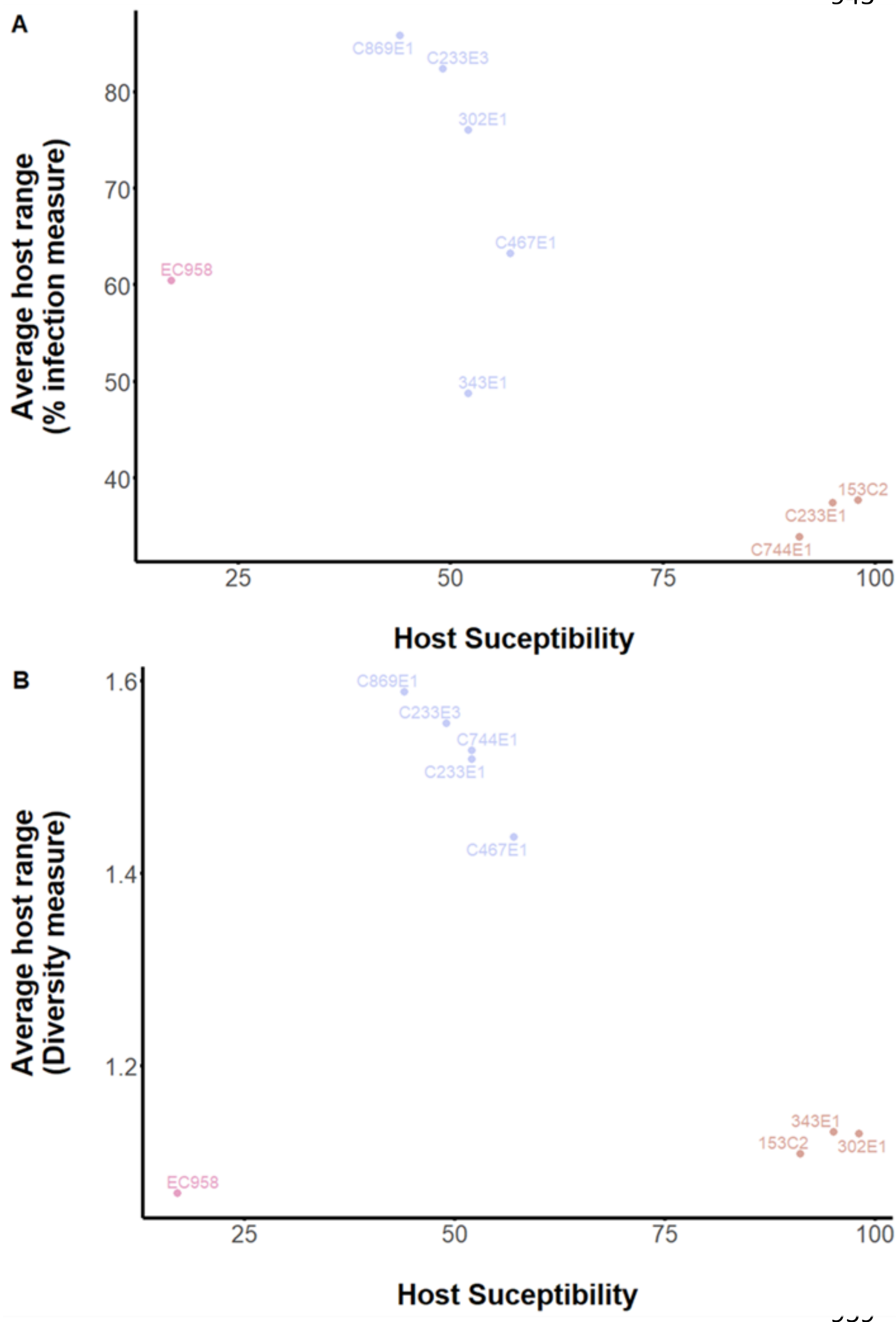


**Figure 5.** The relationship between the susceptibility of nine ST131 hosts to phage infection plotted against the average host range of phages capable of infecting each strain. Host susceptibility was defined as the number of phages (out of the 116 isolated) capable of infecting each host strain. **A.** Host range represented by average % infection (the % of the 9 ST131 hosts the phage can infect). **B.** Host range represented by the diversity measure of host range. The name of each ST131 host is annotated in the colour that corresponds to the host grouping they are part of (A, B, C).

960 ## Broader host range phage do not demonstrate lower infection
961 ## efficiency

962

963 A Pearson's product moment correlation was carried out to detect whether there
964 was any evidence of a trade-off occurring between the breadth of a phages host
965 range (the % of our 9 hosts that a phage can infect) and the level of infectivity of
966 on each host (PFU/mL) (Figure 6). No correlation was found between host range
967 and the average PFU/mL (Log 10) of phage (Figure 6, $t=0.35$, $df=111$, $P>0.05$).
968 Host range was represented as proportion of ST131 strains infected due to the
969 fact that the measure of PFU/mL is a component in the calculation of host range
970 using our novel diversity index measure. A quadratic regression was fitted to this
971 data with no effect.

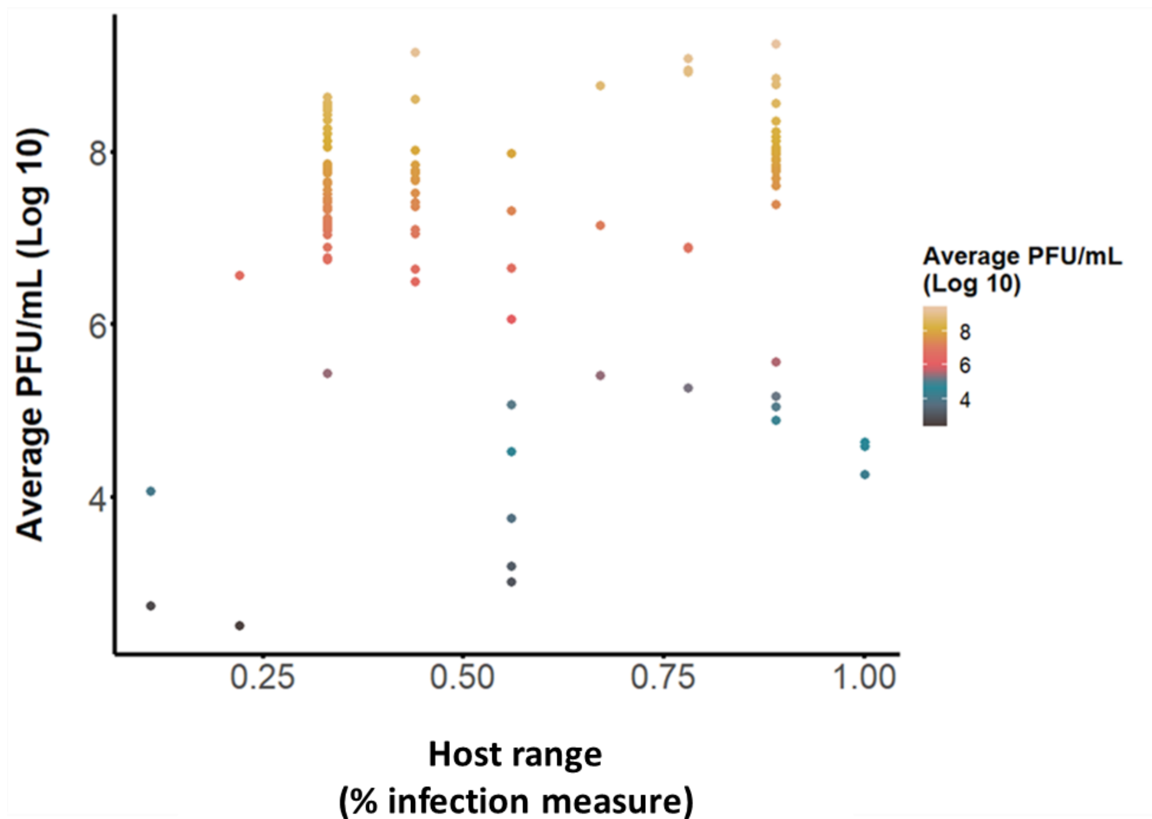


**Figure 6.** Correlation between the average PFU/mL ($Log_{10}$) of phages isolated and the host range (measured using % infection method) of phages. Correlation conducted to determine whether there is evidence of a trade-off between the number of hosts infected (breadth of host range) and the level of infection (PFU/mL) on these hosts. No correlation was identified. Host range was represented using the % infection method as PFU/mL is used to calculate our diversity measure of host range.

972 # Discussion
973
974
975 Over the course of this project, we were able to assess the benefits of a novel
976 method for quantifying bacteriophage host range. Typically, host range is
977 measured as the taxonomic diversity of hosts that a phage can successfully infect
978 (de Jonge et al., 2019). Ecological measures of diversity incorporate two
979 dimensions - number of species (species richness) and their evenness in term of
980 abundance (Stirling and Wilsey, 2001). Traditional measures of bacteriophage
981 host range are one dimensional, only reporting on the breadth of host range
982 (number of host strains that can be infected) and fail to provide any quantitative
983 detail on the degree of phage infectivity (PFU/mL). Spot testing is the most
984 common method used to determine the host range of bacteriophages and
985 through the counting of individual plaques can provide valuable information on
986 infection rate on any given host. Infection rate is an important aspect of phage
987 therapy applications as, due to their self-propagation, any initial inoculum will
988 undergo multiple rounds of infection and transmission in target bacteria (Putra
989 and Lyrawati, 2020). As infection efficacy in vitro can provide an indication of
990 efficiency of phage propagation in vivo, it is an important population dynamic
991 parameter that should be considered in the process of characterising host range
992 (Fernandez et al., 2019; Antoine et al., 2021). It is however important to note that
993 measurements of PFU/mL obtained in standardised, carefully controlled
994 laboratory conditions may not be reflective of what will happen in more complex
995 in vivo settings. Our novel method uses both degree of infectivity and number of
996 hosts infected, in the same way that diversity measures incorporate species
997 richness and abundance. By including data on the level of infectivity, this measure

998 of host range will provide a better indicator of phage fitness across a range of
999 hosts.

1000 Throughout this study we have carried out comparisons of the results of
1001 analyses conducted using a traditional percent infection measure versus our
1002 novel diversity measure of phage host range. These comparisons provide a
1003 significant and consistent indication of the benefit of our novel method in
1004 identifying patterns in phage host range that would otherwise go undetected.
1005 Some of these patterns can be observed in Figure 1 which visualises the 116
1006 phages capable of infecting *E. coli* ST131 isolated during this study. This
1007 heatmap illustration of our phage collection allows us to clearly visualise the
1008 diversity that exists amongst these phages in their patterns of infectivity across
1009 all nine hosts but also provides a direct visual comparison of the additional
1010 information that can be acquired using our more complex measure of host range.
1011 Figure 1A incorporates the level of infectivity of each phage on each host strain
1012 whereas Figure 1B represents infectivity on a binary yes/no basis. What is evident
1013 is that phage that are indistinguishable in Figure 1B do in fact possess distinct
1014 differences in their infectivity (Figure 1A) that may well translate into differences
1015 in their effectiveness as phage therapy candidates. The benefits of this method
1016 are further substantiated by Figure 2 which depicts the results of spot assays of
1017 two distinct phages. If we used the % infection measure, the host range of these
1018 two phages would be identical yet Figure 2 clearly demonstrates that these
1019 phages differ in their infective abilities. Whilst the % infection method of host
1020 range would deem both phages as equally valuable, our modified method is able
1021 to detect important variations between phage that may have substantial
1022 consequences on their suitability as therapeutic agents. Our modified measure
1023 of host range is thus able to successfully detect diversity amongst phages that

1024 may have otherwise been overlooked. Identifying patterns in this diversity may
1025 provide a useful indication as to how we can achieve the preferential isolation of
1026 phages most suited to clinical use.

1027 One of the most striking patterns in this study is the relationship between the
1028 level of infectivity and breadth of host range. The 116 phages isolated as part of
1029 this study appear to possess one of three distinct patterns of host range that can
1030 be visualised in Figure1 (1,2,3). The block at the top of the figure is composed of
1031 phage that possess the broadest host range with the majority of phages capable
1032 of infecting 8/9 of our panel of hosts. The second block situated below this is
1033 composed of phage that possess slightly narrower host ranges that are largely
1034 restricted to the infection of isolation hosts belonging to Group B. The separation
1035 of isolation hosts into groups A, B and C has been directed by patterns in phage
1036 infection that can be observed in Figure 1. These groupings are likely to result, at
1037 least somewhat, from the degree of relatedness between hosts and we therefore
1038 acknowledge that the results of our study may be limited by phylogenetic effects.
1039 The results of genetic sequencing analysis presented in Table 2 provides support
1040 for this, with the similarity among phages in the characteristics of host range,
1041 contig length, viral family and gene count strongly related to the grouping that
1042 their isolation host belongs to, with all phages isolated using group B hosts
1043 belonging to the family Siphoviridae. Interestingly, these phages appear to have
1044 the highest levels of infectivity denoted by the darker colouration of the heatmap
1045 blocks in Figure 1A. The fact that high infectivity is largely confined to phage that
1046 possess an intermediate breadth of host range suggests a trade-off between the
1047 level of infectivity and the number of hosts a phage infects. The existence of such
1048 a relationship is supported by Figure 6 which concludes that phage with
1049 intermediate host range possess higher levels of infectivity. The sequencing

1050 results depicted in Table 2 indicate that these different groups of phages have a
1051 taxonomic basis, so phylogenetic effects rather than quantitative trade-offs may
1052 shape this relationship. Nevertheless, within the limits of this study, high infectivity
1053 is a trait that is restricted to phage that infect fewer hosts. This pattern would not
1054 have been detected if it was not for the use of our more complex measure of host
1055 range. Based on percent infection measures where the value of a phage is
1056 determined solely by the number of hosts it can infect, many of the phage that
1057 possess intermediate host range may have been discarded as potential
1058 therapeutic agents. However, with the use of our novel measurement, we have
1059 identified that these phages outperform their broad host range counterparts in
1060 their level of infection on each host. There may be circumstances in which the
1061 therapeutic application of phage that possess high replication rates on a specific
1062 target host is beneficial and, as such, being able to identify the interplay between
1063 the breadth and depth of host range will be of use. The use of measures that
1064 allow us to achieve the most detailed and data rich characterisation of phage host
1065 range, a property vital to their clinical application, will be vital for the progression
1066 of phage therapy as an antimicrobial strategy and we therefore recommend the
1067 uptake of our method across the field.

1068 The results of whole-genome-sequencing (WGS) of a selection of phage
1069 isolates reveals the clustering of genetically similar phage by isolation host used.
1070 Table 2 demonstrates that the same or similar phage can be isolated from a range
1071 of sampling environments. For example, phages that share an identical or similar
1072 contig length, family, estimated characterization and gene count were found
1073 across wastewater, clinical and sludge environments (Table 2). This suggests
1074 that phages that possess the same or similar genetic backgrounds are not found
1075 exclusively in specific sewage environments. This may be related to the

1076 permeability of sewage environments as well as the spatial and temporal variation
1077 associated with them. In contrast, the genetic background of phage appears to
1078 cluster based on the ST131 host strain used for isolation. For example, phage
1079 isolated using group B hosts share a contig length of between 44-45kbp, are all
1080 identified as members of the bacteriophage family Siphoviridae, possess gene
1081 counts between 72-76 and were all assigned to the taxonomic label of '*E. coli*
1082 virus Golestan'. If these genetic characteristics can be demonstrated to
1083 correspond to the usefulness of a phage for phage therapy, then the relatedness
1084 between isolation host and the types of phages isolated may be of great use for
1085 optimising phage isolation protocols.

1086 *E. coli* phage VB_EcoS-Golestan is a member of the Siphoviridae family and
1087 belongs to the genus *Kagunavirus* (Schoch et al., 2020). The characteristics and
1088 genome of VB_EcoS-Golestan has been investigated extensively by Yazdi et al.,
1089 (2020). Although the primary focus of this study is on the characteristic of phage
1090 host range, there are other factors that contribute to the therapeutic potential of
1091 a phage including their lytic ability, possession of genes encoding virulence
1092 factors, latency period and burst size (Fernandez et al., 2019; Mirzaei and
1093 Nilsson, 2015). Due to the relationship between the burst size of a phage and its
1094 propagation in its host, a large burst size, combined with a short latency period,
1095 are good characteristics in phage therapy candidates (Yazdi et al., 2020; Sharma
1096 et al., 2021; Abedon, Herschler and Stopar, 2001). With a latent period of 40
1097 minutes and burst size of 100 PFU per cell, VB_EcoS-Golestan complies with
1098 this (Yazdi et al., 2020). It was also demonstrated to possess a broad host range
1099 against both antibiotic sensitive and MDR uropathogenic *E. coli* isolates and
1100 whole genome annotation confirmed the absence of virulence factors encoded in
1101 its genome. The significance of these findings stems from the high homology of

1102 this phage with other *E. coli* phages belonging to the Siphoviridae family. This
1103 high homology suggests that the characteristics described by Yazdi et al. (2020)
1104 may be shared by several phages isolated within our study which would indicate
1105 we have been successful in isolating phages with high potential for successful
1106 application as therapeutic agents. The WGS, and taxonomic classification of
1107 bacteriophage isolates that is subsequently made possible, can therefore be
1108 used to infer features of phage that provides an indication of their potential as
1109 agents for phage therapy.

1110 An understanding of the factors responsible for the diversity amongst phage
1111 observed in Figures 1 and 2 may help to enhance the success of isolation
1112 attempts. For the purposes of this study, we set out to explore whether the
1113 isolation environment or isolation host strain were significant drivers of the
1114 diversity observed in phage host range. It is widely accepted that sewage and
1115 sewage-contaminated environments are some of the best-known places to hunt
1116 for bacteriophages, including those capable of infecting clinically relevant
1117 pathogens such as *E. coli* ST131 (Aghaee et al., 2021). Throughout the literature
1118 numerous examples of this can be found such as the isolation of a novel
1119 bacteriophage from wastewater samples in Iran (Yazdi et al., 2020). The phage,
1120 labelled VB_EcoS-Golestan, was identified as capable of successfully infecting
1121 MDR uropathogenic *E. coli* isolates sampled from hospital patients suffering with
1122 UTIs and as such represents a potential candidate for the use of phage therapy
1123 in the treatment of such infections (Yazdi et al., 2020). It is important to recognise
1124 that a high level of diversity exists in the array of sewage environments that may
1125 be used for phage isolation ranging from municipal wastewater to agricultural
1126 sewage sludge. Subtle differences between these sewage subtypes can be

1127 important determinants of the abundance of phage as well as of the properties
1128 that these phages possess (Van Charante et al., 2021).

1129 To identify such differences and maximise the likelihood of isolating ST131-
1130 specific phages, we took samples from four distinct sewage environments
1131 referred to throughout this publication as clinical sewage, agricultural waste,
1132 community wastewater and activated sludge. We hypothesised that clinical
1133 sewage would be the most successful sampling environment for the isolation of
1134 ST131 targeting phages due to the high abundance of MDR associated with
1135 healthcare settings (Chia et al., 2020). Indeed, *E. coli* is one of the most
1136 frequently isolated MDR pathogens in clinical environments with ST131 known to
1137 be a causative agent of several nosocomial infections including UTIs,
1138 septicaemia, and neonatal meningitis (Wang et al., 2019; Fahim, 2021; Johnson,
1139 2005). The widespread use of antibiotics in clinical settings, also acts to exert a
1140 strong and continuous selective pressure that promotes the emergence and
1141 persistence of antibiotic resistance. In fact, hospital wastewater is reported to
1142 contain as much as 100 times higher antibiotic levels than community sewage
1143 treatment plants (Baquero, Martínez and Cantón, 2008; Kummerer, 2004). These
1144 higher concentrations of antibiotics promote the evolution of resistance among
1145 bacterial strains, such as ESBL *E. coli*, that cooccur in the wastewater system.
1146 This is evidenced by a study that found that *E. coli* strains isolated from hospital
1147 wastewater were resistant to significantly more antibiotics than those isolated
1148 from municipal sewage treatment plants (Katouli et al., 2012). Combined, these
1149 factors indicate that clinical sewage presents an ideal environment for the
1150 isolation of phages capable of infecting MDR strains such as ST131.

1151 Measures of the abundance of ST131 across all four sewage environments
1152 failed to support the hypothesis that clinical sewage is a superior isolation

1153 environment for the acquisition of ST131-specific phage (Figure 3B). Due to
1154 phages obligate requirement for a bacterial host, the abundance of ST131 was
1155 used as a proxy for the presence of ST131-targetting phage (Clokie et al., 2011).
1156 Whilst general coliphage abundance did not differ significantly between
1157 environments (Figure 3A), the abundance of ST131 did (Figure 3B), with no
1158 evidence of ST131 present in clinical or agricultural sewage. Although this may
1159 be due to the incomplete sampling of clinical sewage environments, it is a result
1160 mirrored by other studies such as Melo et al. (2014). Owing to the prominent role
1161 of *Staphylococcus epidermidis* in nosocomial infections, Melo et al. (2014)
1162 hypothesised that *S. epidermidis* specific phages would be found in clinical
1163 sewage, but sampling attempts failed to support this. The parallels seen in this
1164 study suggests that external factors associated with clinical sewage may be
1165 influencing the occurrence and viability of phages in these environments
1166 (Jonczyk et al., 2011). One possibility is that phages may be inactivated during
1167 their exposure to chemical disinfectants regularly used in clinical settings (Agún
1168 et al., 2018). This hypothesis was investigated by Agún et al. (2018) who
1169 demonstrated that overnight incubation with four distinct disinfectants led to the
1170 inactivation of an anti-staphylococcal phage. The study also evidenced the ability
1171 of some disinfectants to interfere with the efficacy of the phage to establish an
1172 infection (Agún et al., 2018). This may be a result of a direct impact on the
1173 physical properties of the phage that are crucial to their antimicrobial activity, or
1174 a result of the impact of disinfectants on the availability of viable hosts.
1175 Regardless of the mechanisms involved, it seems likely that the interactions that
1176 arise between host, phage and the chemical agents they are exposed to may go
1177 some way to explaining the unsuccessful isolation of phages from clinical sewage
1178 environments that is echoed throughout the literature. To aid future sampling

1179 attempts, further investigation into the susceptibility of phages to commonly used
1180 disinfectants is required. In the meantime, a focus on sampling environments with
1181 lower levels of chemical contamination may result in the more successful isolation
1182 of viable phage.

1183 Together with phage abundance, measurements of phage host range must be
1184 considered in the comparison of isolation environments. Although phage
1185 abundance can provide a useful indication of where to focus initial sampling
1186 attempts, it can be of limited value when used as an independent measure. When
1187 the intended application of phage is for therapeutic use, the value of sampling
1188 environments is largely determined via measurements of host range due to the
1189 generalised preference for broad host ranges. As a result, our assessment of the
1190 effectiveness of sewage environments was driven by the average host range of
1191 phages isolated (Figure 3C) and concluded that phages isolated from community
1192 wastewater possessed significantly broader host ranges than those isolated from
1193 all other sewage subtypes tested (Figure 3C).

1194 Ecological factors such as host density and diversity may provide some
1195 explanation for the preferential selection of broad host ranges in community
1196 wastewater environments. The term bacteriophage translates directly as ‘bacteria
1197 eater’ (Stone et al., 2019). A broad host range phage can be likened to a
1198 generalist predator, that is, one that can thrive in a wide variety of environmental
1199 conditions and make use of different resources. Ecological parameters, such as
1200 host density and host diversity, have been identified as likely determinants of the
1201 probability of finding broad host range phage in any given environment (de Jonge
1202 et al., 2019). Experimental evidence suggests that broad host range seems most
1203 likely to be selected for in environments where cell densities are low, but species
1204 diversity is high (Dekel-Bird et al., 2015). Low cell density drives high competition

1205 for host resources which promotes the evolution of host range expansion which
1206 is only possible if species diversity is high, and phages have alternative hosts to
1207 infect. Wastewater treatment plants process sewage from entire communities
1208 meaning the resulting material can be described as a 'pooled fecal sample'
1209 representing the gut communities of several hundred or thousand individuals.
1210 The more individuals included in a sample means more bacterial diversity is
1211 represented which would suggest that wastewater treatment plants should be
1212 environments where the requirement of high species diversity is met. Indeed,
1213 there is evidence that phages isolated from pooled fecal samples possess a
1214 broader host range than those isolated from manure samples taken from a small
1215 set of individuals (Akhtar, Viazis and Diez-Gonzalez, 2014). In addition, cell
1216 densities are likely to be lower in community wastewater environments. This
1217 comes as a direct by-product of the fact that the material is pooled from many
1218 individuals which acts to heavily dilute the sampled material. These results
1219 suggest that variation in the ecological conditions of sewage environments may
1220 be responsible for the differential selection of phage host range. Successful
1221 identification of the factors that promote broad host ranges will allow us to steer
1222 isolation attempts more accurately towards the acquisition of phage with the most
1223 promising therapeutic potential.

1224 In this study isolation host used was also a significant determinant of the host
1225 range of phages with the use of certain isolates resulting in the more consistent
1226 isolation of broad host range phage (Figure 3C). Unlike the clear effect of
1227 sampling from community wastewater environments, no single host strain was
1228 independently accountable for the isolation of phage with significantly broader
1229 host range. In fact, rather than acting independently, the 9 hosts used appear to
1230 cluster into three distinct groups (A, B, C). Interestingly, 54.4% of phages that

infect at least one isolation host belonging to group C only infect isolation hosts belonging to this group. This suggests that the use of a single host strain is likely to restrict isolation attempts to the acquisition of phages with narrow host ranges and so to achieve the preferential isolation of broad host range phage, the concurrent use of multiple isolation hosts is recommended. Indeed, although the use of a single host is commonly seen throughout the literature, evidence points strongly towards the use of multiple bacterial hosts to help to achieve more consistent isolation of broad host range phage (Ross, Ward and Hyman, 2016). In order to determine the most effective combination of hosts to use, we must first attempt to explain the effect of isolation host on phage host range.

Figure 4 shows the influence of isolation host on the host range of phages isolated. Informed by patterns identified throughout this study (Figure 1; Figure 4; Figure 5), we divided our panel of 9 ST131 hosts into three groupings referred to as group A, B and C. These groupings were determined based on the similarity of host strains in the host ranges of the phages they isolate. The variation within these groups in the values of host range is considerably smaller when using our modified measure of phage host range which allows informative patterns in phage host range to be identified more clearly (Figure 4B). Through statistical analysis we were able to confirm that there were in fact significant differences between these groupings, particularly between group B and C with hosts belonging to group B more commonly isolating phage that possess a broader host range than those isolated by group C hosts. All 9 of the isolation hosts used in our study had been previously confirmed, by PCR and sequence analysis, as unique ST131 isolates (Anne Leonard and colleagues, unpublished data). The host groupings identified are likely to be determined to some extent by differing levels of genetic relatedness amongst these strains. Using unpublished sequence data provided

1257 by Anne Leonard and colleagues, we were able to provide support for this with
1258 distinct genetic differences present between group B and group C hosts in the
1259 number of insertion sequences, the number of virulence and antibiotic resistance
1260 genes, the number and type of plasmid as well as the presence or absence of
1261 class 1 integrons. Gaining a better understanding of the features of a host that
1262 determines its influence on host range will be important if we are to encourage
1263 the isolation of broad host range phage. It is important to consider that a lack of
1264 understanding of the influence of isolation host on the host range of phage poses
1265 a threat to the validity of conclusions reached on the effect of other factors such
1266 as the type of environment phage are isolated from. This is because it is not
1267 possible to investigate the influence of these factors independent of the effect of
1268 isolation host as in any isolation process, a host is a necessary requirement. For
1269 example, the use of a single isolation host from group C that biases isolation
1270 attempts towards narrow-host range phages may inadvertently limit the diversity
1271 of phage isolated from a particular sampling environment. This emphasises the
1272 importance of studies such as ours in determining the effect of isolation host on
1273 phage host range as well as the importance of future studies in exploring the
1274 mechanisms behind it. Interestingly Yu, et al. (2016) found that it is not just the
1275 selection of, or number of, host strains used for isolation that influences host
1276 range but also the method by which these hosts are employed. They found that
1277 the sequential exposure of phage samples to multiple isolation hosts in a specific
1278 circular sequence led to the most successful isolation of polyvalent, or broad host
1279 range, phage. This, alongside the evidence provided by our study demonstrate
1280 the multiple opportunities and ways in which it is possible to manipulate the
1281 outcome of phage isolation attempts. Exploiting this knowledge will allow us to

1282 shift the bias commonly present in isolation methods towards the preferential
1283 isolation of broad host range phage (Yu et al., 2016).

1284 The susceptibility of hosts to phage infection may influence the host range of
1285 the phages they isolate. A meta-analysis of host range indicated that there is a
1286 nested structure to host-phage interactions (Flores et al. 2011). That is,
1287 generalist, or broad host range, phage tend to infect the most resistant bacterial
1288 strains whereas specialist, or narrow host range, phage infect hosts that are the
1289 most susceptible to infection (Flores et al., 2011). This suggests that diversity in
1290 the susceptibility of hosts to phage infection contributes to the variation observed
1291 in phage host range and that the use of 'hard to infect' host strains may allow us
1292 to achieve the targeted isolation of broad host range phage (Hibbing et al., 2010).
1293 Analysis of the host susceptibility of all nine ST131 strains used in our study
1294 provides some support for this. If broad host range is indeed a result of trade-offs
1295 between the ease of infection and competition for hosts, one would expect the
1296 strains that most consistently isolate broad host range phage, to be infected, on
1297 average, by fewer phages. Figure 5 shows the percentage of phages in our
1298 collection able to infect each host (host susceptibility) plotted against the average
1299 host range of phages isolated using each host strain. Although the relationship
1300 between host susceptibility and host range is not significant when using our
1301 diversity-based measure of host range, this is likely a result of the outlying data
1302 point representing EC958. If we exclude this strain, the pattern generated by the
1303 data is that hosts infected by fewer phage, isolate phage with the highest average
1304 host range (Figure 5B). This is corroborated by the significant negative correlation
1305 between host range and host susceptibility when using our percent infection
1306 measure of phage host range (Figure 5A). If we use host susceptibility as a proxy
1307 for host resistance to phage infection then our data, with the exception of EC958,

1308 suggests that the use of 'hard to infect' host strains promote the isolation of broad
1309 host range phage and thus provides support for the nested structure to host-
1310 phage interactions described by Flores et al. (2011). Figure 5A and 5B effectively
1311 demonstrates the higher degree of precision available in detecting such patterns
1312 in our dataset when using our modified measure of host range as opposed to the
1313 use of a basic % infection method. In Figure 5B, hosts clearly cluster in the same
1314 distinct groupings as previously described providing a much more distinct
1315 visualisation of the relationship between host susceptibility and phage host range
1316 and yet further support for the use of our modified measure of host range. In order
1317 to exploit the influence that isolation hosts appear to exert on phage host range,
1318 further studies should explore what it is that defines a host as 'hard to infect'.

1319 If we are to harness broad host range to improve the effectiveness of phage
1320 therapy, we must first seek to understand the consequences associated with host
1321 range expansion. The benefits of broad host range are clear, with phages that
1322 exploit multiple hosts as effectively as other phage infect a single host, in
1323 possession of an evolutionary advantage. This raises the question of why
1324 generalists have failed to dominate communities and instead co-exist alongside
1325 specialists (Bono, Draghi and Turner, 2020). The most obvious explanation is the
1326 existence of a fitness cost associated with generalism which aligns with the
1327 classical theory that a 'jack-of-all-trades' is a master of none (Levin, 1968; Lynch
1328 and Gabriel, 1987). As such, phages trade the increased breadth of hosts they
1329 can infect with a reduction in their mean performance across these hosts
1330 (Kassen, 2002). We explored whether such trade-offs could be detected within
1331 our data set by analysing whether there was any relationship between phage host
1332 range and their average level of infectivity, measured by PFU/mL (Figure 6). We
1333 found no evidence of these trade-offs with generalist phage seemingly as

1334 effective at exploiting their hosts as their narrow host range counterparts (Figure
1335 6). As the existence of 'no-cost' generalists is highly unlikely, the lack of evidence
1336 of the fitness costs of host range expansion in our study does not suggest they
1337 do not exist but indicates they are experienced in ways other than the reduction
1338 in infective ability, that we predicted would be observed. For example, a study by
1339 Bono, Draghi and Turner (2020) suggests that broad host range phage may in
1340 fact experience a fitness cost through reduced evolvability. That is, they are less
1341 able to adapt and respond to inevitable spatial and temporal changes in host
1342 availability. Improving our understanding of the fitness costs associated with a
1343 broad host range phenotype is vital if they are the preferred target of isolation for
1344 therapeutic use. An understanding of how and why these costs occur, may allow
1345 us to find ways to ameliorate them and thus encourage the evolution of the broad
1346 host range phage that are desired for clinical use.

1347 The purpose of this study was to help improve methods of isolating phage,
1348 specifically those able to infect strains belonging to the MDR genotype *E. coli*
1349 ST131. We found that isolation host and environment were significant factors that
1350 influenced the success of isolation attempts. As an important determinant of the
1351 usefulness of phage for therapeutic purposes, this success was measured using
1352 the characteristic of host range with broad host range phage the preferred targets
1353 of isolation. The isolation of broad host range phage is of particular relevance due
1354 to the complex subclonal structure of the ST131 complex (Lopes et al., 2021;
1355 Nicolas-Chanoine, Bertrand and Madec, 2014). The development of our novel
1356 measure of phage host range, and our relatively consistent demonstration of its
1357 advantage over traditional measures, is an important outcome of this study. For
1358 the application of phage for therapeutic purposes, it is important to attain the most
1359 complete measure of phage host range as this characteristic relates directly to

1360 the effectiveness of phage as potential antimicrobial agents. Our novel method
1361 allows us to achieve this by accounting for variation amongst phage in their level
1362 of infectivity, which is a commonly overlooked, yet informative, aspect of phage
1363 host range. Within the limits of our study, phages isolated from community
1364 wastewater environments possessed a significantly higher host range. Identifying
1365 the environmental characteristics that are responsible for driving this relationship
1366 will help contribute to the development of a more systematic and methodical
1367 approach to the assessment of the usefulness of sampling environments for the
1368 isolation of broad host range phage. The isolation host used was also a significant
1369 determinant of phage host range with certain ST131 isolates resulting in the
1370 preferential isolation of broader host range phage. Our data suggest that contrary
1371 to the commonly used practice of utilising a single isolation host, the use of a
1372 panel of isolates capable of isolating phage with disparate host ranges, will
1373 provide us with a collection of phages that, together, offer comprehensive
1374 antimicrobial protection against a wide range of isolates. Finally, there are several
1375 suggestive patterns within our data set that provide promising indications of ways
1376 in which our isolation methods may be optimised to achieve the preferential
1377 isolation of broad host range phage. These include, but are not limited to, the use
1378 of hosts belonging to specific phylogenetic backgrounds or that possess
1379 intermediate susceptibility to phage infection. Although not the primary focus of
1380 this study, we recommend that these patterns form the basis of future research.

1381 The main objective of this research has been the isolation of ST131 specific
1382 bacteriophage. However, if the features of isolation environments and hosts that
1383 are responsible for the promotion of broad host range are relevant to all phage,
1384 regardless of their host, the conclusions of this and future studies will be
1385 applicable to other MDR pathogens of concern. Although unlikely that the future

1386 of phage therapy will rely upon the isolation of broad host range phage directly
1387 from the environment, the evidence provided by studies such as ours will be
1388 pivotal in achieving the biotechnological advancements that are predicted to
1389 dominate the field of phage biology (Pires et al., 2020). Detail on the factors
1390 promoting broad host range and the methods by which we might optimise it
1391 artificially will be utilised to evolve broad host range phage in vitro in a rapid,
1392 efficient, and clean approach that primes them for clinical use. Although many
1393 barriers to the therapeutic application of lineage-specific phage remain in place,
1394 including the evolution of phage resistance and in vivo tolerance of bacteriophage
1395 preparations, our research provides great promise. Above all else, we have
1396 demonstrated the ease at which phage capable of infecting a critical priority
1397 pathogen can be isolated from the environment, which contributes greatly to the
1398 mounting evidence in support of the use of these naturally occurring antimicrobial
1399 weapons as a solution to the antibiotic crisis. The progression of phage therapy
1400 that ensues from this support will allow us to remain able to successfully treat
1401 patients suffering from life-threatening antibiotic-resistant infections. More
1402 importantly, it will allow us to avoid returning to the levels of mortality and
1403 morbidity experienced prior to the discovery of antibiotics, something that without
1404 the intervention provided by phage, remains a very real threat worldwide.

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# Chapter two

## The combined use of probiotic *E. coli* with lineage-specific bacteriophage to achieve the targeted elimination of ST131

### Introduction

The elimination of intestinal reservoirs of MDR clones may provide an effective strategy for the control of the clinical infections that they are associated with (Sarkar et al., 2018). ST131 is widely recognised for its proficiency in effective colonisation of, and long-term persistence in, the gastrointestinal niche (Muller, et al., 2021; Sarkar et al., 2018). Although the mechanisms responsible for the enhanced colonisation ability of ST131 require further investigation, the production of type 1 fimbriae and the ability to overcome microbiota-mediated colonisation resistance have been identified as contributing factors (Sarkar et al., 2018). Concerningly, the prolonged and typically asymptomatic carriage of ExPEC strains, such as ST131, has been identified as a prerequisite for the development of clinically important infections such as UTIs and bacteremia. For example, uropathogenic isolates found in the urine of patients suffering from UTIs are frequently found in the fecal samples taken from patients at the time of infection indicating that the intestinal carriage of such isolates is an important upstream step in the development of these infections (Vimont et al., 2012; (Johnson, Johnston and Gordon, 2017). A similar association has been identified by Tacconelli et al. (2019) who reported that among 497 hematological patients,

1429 the most important risk factor for the development of bacteremia was previous
1430 colonisation with ESBL-producing Enterobacteriaceae (Tacconnelli et al., 2019).
1431 However, the risk of developing these sporadic, and often untreatable, infections
1432 is just one of the many concerns associated with the intestinal carriage of MDR
1433 organisms (Bernasconi et al., 2020). Other potential consequences include
1434 increased environmental spread as well as increased rates of person-to-person
1435 transmission within communities (Bernasconi et al., 2020). In addition, prolonged
1436 carriage of such strains has the potential to result in the horizontal spread of
1437 resistance genes to non-pathogenic members of the gut microbiome (Bernasconi
1438 et al., 2020). The global rise in MDR infections attributed to *E. coli* ST131 is
1439 inextricably linked to the epidemic of the invisible and silent intestinal colonisation
1440 of ST131 in the community (Sarkar et al., 2018; Johnson, Johnston and Gordon,
1441 2017). To combat this, the development of interventional strategies designed to
1442 target the intestinal reservoir of MDR clones are required (Sarkar et al., 2018).

1443 One such method is decolonisation therapy defined as 'any measure that leads
1444 to loss of detectable multidrug-resistant gram-negative bacteria (MDR-GNB)
1445 carriage at any site' (Tacconelli et al., 2019). Through the eradication or reduction
1446 in the asymptomatic carriage of ST131 we aim to reduce the incidence, and
1447 recurrence, of the MDR clinical infections it is associated with (Rieg et al., 2015;
1448 Vimont et al., 2012; Tacconelli et al., 2019). The concept of decolonisation is not
1449 a novel one and has been used to inform the development of selective
1450 decontamination of the digestive tract (SDD), an infection prevention measure
1451 used for intensive care patients in countries such as the Netherlands (Wittekamp,
1452 et al., 2019; Elderman et al., 2021). SDD is a strategy that uses nonabsorbable
1453 antibiotics, most commonly colistin, to decrease the risk of infection by gram-
1454 negative pathogens (Rieg et al., 2015). An example of this is provided by Oren,

1455 et al. (2013) who successfully achieved the eradication of carbapenem resistant
1456 Enterobacteriaceae from the gastrointestinal tract through the combined use of
1457 colistin and gentamicin. However, the same obstacles posed by the emergence
1458 of antibiotic resistance, as well as the unintentional collateral damage are faced
1459 when utilising this method. In recent years a new approach has been proposed
1460 that capitalises on the specific, self-propagating characteristics of bacteriophage
1461 to selectively remove MDR pathogens from the intestinal reservoir (Poirel et al.,
1462 2020). Despite being in its infancy, reported successes of decolonisation with
1463 phage demonstrate great promise such as the decolonisation of a MDR and
1464 carbapenemase-producing *Klebsiella pneumoniae* strain that had persisted in a
1465 patient for over 6 months (Corbellino et al., 2020). Promisingly, the efficacy of
1466 phage, typically administered as a cocktail formulation, in reducing the
1467 abundance of *E. coli*, is comparable to the reduction achieved by antibiotics such
1468 as ampicillin and ciprofloxacin (Dissanayake et al., 2019; Cieplak et al., 2018).
1469 The use of phage for decolonisation is unlikely to be used as a standalone
1470 treatment but will aim to lower frequencies of the target pathogen to levels that
1471 can be managed by the hosts immune system or by alternative antimicrobials. To
1472 reduce our reliance on antibiotics, the use of probiotics has been proposed to
1473 achieve such objectives.

1474 The World Health Organisation (WHO) defines probiotics as 'live
1475 microorganisms that, when administered in adequate amounts, confer a health
1476 benefit to the host' (Hotel and Cordobda, 2001). Probiotics can provide these
1477 benefits via several mechanisms including the regulation and stimulation of the
1478 host immune response, the production of antibacterial toxins known as microcins
1479 and via competition with pathogens for adhesion and essential nutrients (Yan and
1480 Polk, 2011; Lehtoranta, Latvala and Lehtinen, 2020; NASPGHAN Nutrition

1481 Report Committee et al., 2006). For the purposes of this study, the main
1482 mechanism of interest is that of ecological competition between probiotic and
1483 pathogenic strains in the gastrointestinal niche. Freter (1992) argued that
1484 although a multitude of factors affect the ability of an organism to achieve
1485 effective colonisation, 'competition for nutrients is paramount for success in the
1486 intestinal ecosystem' (Conway and Cohen, 2015). Considering the importance of
1487 resource competition in driving the structure of ecosystems, Freter (1992)
1488 suggested that the utilisation of probiotic strains to outcompete pathogens for
1489 essential nutrients may prove to be an effective therapeutic strategy (Conway
1490 and Cohen, 2015). The use of probiotics in this way is referred to as bacterial
1491 interference defined as 'the use of bacteria of low virulence to compete with and
1492 protect against colonisation and infection by disease-causing organisms'
1493 (Darouiche and Hull, 2012). Although from a theoretical point of view, there is
1494 considerable potential for the preventative and therapeutic role of probiotics,
1495 much of the research conducted has failed to support this, instead serving as
1496 examples of the rather limited and unreliable effects of probiotic application
1497 (Lerner, Shoenfeld and Matthias, 2019). For example, although the
1498 administration of probiotics has been proposed as a method of eliminating MDR
1499 bacteria from the intestinal niche, experimental data in support of this approach
1500 is lacking (Ljungquist et al., 2020). This is exemplified by a study conducted by
1501 Mourand et al. (2017), who found that the administration of two probiotic *E. coli*
1502 strains (ED1a and Nissle) to a sample of pigs, was found to have no significant
1503 impact on the gut carriage or survival of cefotaxime resistant Enterobacteriaceae.
1504 Similarly, as one of the most common bacterial infections globally and a
1505 significant cause of mortality resulting from secondary bloodstream infections,
1506 probiotics have been suggested as an alternative to antibiotics in the treatment

of UTIs (Sihra, et al., 2018; NHS England, 2018; Flores-Mireles et al., 2015; Reid and Bruce, 2006). Despite the numerous studies conducted, a Cochrane review carried out in 2015 concluded that there was no significant difference in the risk of developing recurrent UTI for individuals prescribed probiotics in comparison to those receiving a placebo (Schwenger, Tejani and Loewen, 2015). Interestingly, the lack of randomised clinical trials (RCTs) is commonly reported as one of the main obstacles to the formulation of conclusions regarding the safety or efficacy of probiotics (Grin et al., 2013). Although more RCTs are undoubtedly necessary to determine the net effect of probiotics, the results of those that have been conducted are not encouraging such as those of a recent large scale RCT conducted by the Nuffield Department of Primary Care Health Sciences at the University of Oxford. They set out to investigate whether the use of probiotics could help reduce infections in care home residents but found no effect of the daily administration of two probiotics on the number of infections or levels of ABR bacteria present in residents stool samples (Butler et al., 2021). The examples presented above are just a few of the studies whose results call into question the application of probiotics as a standalone therapeutic strategy (Vidal et al., 2010; de Regt et al., 2010). The development of new, multifaceted approaches such as the adjunctive use of probiotics alongside other antimicrobials may help to replace these discouraging results with a renewed confidence in the role of probiotics for the control of MDR pathogens.

Alternative antimicrobial agents can be used to manipulate the composition of the intestinal niche in a way that enhances the colonisation success, and competitive ability, of probiotic strains. A 5-week long study conducted by Tannock et al. (2011) found that the twice daily administration of the probiotic *E. coli* strain Nissle 1917 (Nissle) failed to achieve the eradication of norfloxacin-

1533 resistant *E. coli.* Unsuccessful eradication attempts such as this can be attributed
1534 to the inability of probiotic strains to establish themselves in the gastrointestinal
1535 niche (Sassone-Corsi et al., 2016). Tannock (2003) explains that in complex
1536 microbial communities such as the gut, it is incredibly difficult for microbes to
1537 establish themselves, with all available niches already occupied. In the same way
1538 that gut commensals competitively exclude pathogenic microorganisms
1539 unintentionally introduced into the gut ecosystem, artificially introduced probiotics
1540 are met with the same hostility which can act as a significant barrier to their
1541 proliferation and ensuing ability to outcompete and eradicate pathogenic bacteria.
1542 This barrier can be reduced through the action of other antimicrobials, such as
1543 antibiotics, whereby their broad-spectrum activity and subsequent depletion of
1544 the host microbiome acts to lower the level of colonisation resistance
1545 encountered by probiotic strains (Suez et al., 2018). In addition, the bactericidal
1546 action of other antimicrobials may also be able to tip the outcome of competitive
1547 interactions in favour of probiotic strains via their influence on the relative
1548 frequency of strains within the population. Microbial competition is commonly
1549 mediated by antagonistic toxins known as bacteriocins (Riley, 2011). Classical
1550 theory into bacteriocin based competition predicts that bacteriocin producing
1551 bacteria are at a selective advantage when their relative frequency is high, that
1552 is, the bacteriocin producing genotype is common (Chao and Levin, 1981). As
1553 the frequency of the target bacterium is reduced by the action of antibiotics,
1554 phage or other antimicrobial agents, the relative frequency of the probiotic is
1555 increased. As a result of positive frequency dependent selection and the
1556 competitive advantage now acquired by the probiotic strain, its capacity to
1557 achieve the competitive exclusion of target pathogens is enhanced. Although
1558 probiotics should not be recommended as a single agent for eradication therapy,

they still display great promise which we will aim to explore further through the course of this study (Goderska, Pena and Alarcon, 2018). Although the most commonly used probiotic strains belong to the gram-positive genera Lactobacillus and Bifidobacterium (Behnsen et al., 2013), some gram-negative strains are also employed. The most common of these and the probiotic strain used for the purpose of this study is *E. coli* Nissle (Nissle).

*E. coli* Nissle 1917 (Nissle) (serotype O6:K5:H1) is a non-pathogenic strain that has been utilised as a probiotic for over 100 years (Massip et al., 2019). Its discovery dates back to 1917 when outbreaks of Shigella were responsible for the widespread incidence of diarrheal disease amongst soldiers (Baker et al., 2014). German physician, Alfred Nissle, astutely noticed that one soldier seemed unaffected by the outbreak, developing no signs of intestinal disease and theorised that the soldier was a carrier of an antagonistically strong E. *coli* isolate able to inhibit the growth of pathogenic strains in the intestinal niche (Sonnenborn, 2016). After achieving experimental confirmation of his hypothesis, Nissle went on to manufacture the strain as a therapeutic product named ‘Mutaflor’ which remains available to buy from pharmacies in Canada, Asia, and in some countries across Europe. The attraction of Nissle as a probiotic, stems from its low virulence and possession of a large set of fitness factors that contribute to its enhanced competitive ability (Behnsen et al., 2013; Toloza et al., 2015). In addition to this, Nissle has been widely reported as resistant to the horizontal transfer of DNA thus minimising the risk of its uptake of the CTX-M-15-encoding plasmid associated with ST131 (Mourand et al., 2017; Sonnenborn and Schulze, 2009; Crook et al., 2019; Lerner, Neidhöfer and Matthias, 2017). Two factors that have been strongly attributed to the success of Nissle are the production of microcins and enhanced capacity for iron acquisition (Sassone-

1585 Corsi et al., 2016; Deriu et al., 2013). Microcins are antibacterial peptides that
1586 inhibit the growth of phylogenetically related strains (Behnsen et al., 2013). The
1587 ability of Nissle to outcompete enteric pathogens has been linked to the
1588 production of two specific microcins referred to as MccH47 and MccM (Patzer at
1589 al., 2003). Sassone-Corsi, et al. (2016) showed the importance of these in
1590 competition among *Enterobacteriaceae* in the gut by demonstrating that WT
1591 Nissle was able to significantly reduce the intestinal colonisation of mice infected
1592 with *Salmonella enterica* whereas Nissle mutants unable to secrete microcins,
1593 could not. Another method by which Nissle acts to displace its competitors is
1594 through its enhanced ability to acquire iron (Sassone-Corsi et al., 2016; Deriu et
1595 al., 2013). The possession of multiple iron uptake systems alongside its
1596 resistance to Lipocalin 2, a mechanism by which hosts withhold iron through the
1597 counteraction of certain siderophores, provides Nissle with the ability to scavenge
1598 for this essential micronutrient more effectively that its pathogenic counterparts
1599 (Deriu et al., 2013). Deriu et al. (2013) confirmed the importance of these
1600 mechanisms for the probiotic activity of Nissle by demonstrating that, in contrast
1601 to their WT counterparts, mutants of Nissle deficient in iron uptake mechanisms
1602 fail to reduce the ability of S. *enterica* to colonise the intestine. Evidently, the
1603 probiotic effect of Nissle is strongly related to its ability to directly compete with
1604 pathogens that co-colonise the intestinal niche. The results of the studies
1605 described above as well as the long-standing use of Mutaflor, strongly supports
1606 the use of this probiotic strain as a method to eliminate the carriage of enteric
1607 pathogens from the gastrointestinal tract.

1608 In this study we hypothesize that combined use of phage with probiotic bacteria
1609 will more effectively suppress populations of MDR E. *coli* ST131. This two-tiered
1610 strategy will combine the predatory action of phage with the competitive ability of

1611 Nissle. Although the evolution of resistance to phage will attempt to be mitigated
1612 against through the application of carefully constructed phage cocktails, its
1613 eventual emergence is almost inevitable. This strategy aims to capitalise on
1614 evolutionary trade-offs to achieve the desired outcome of decolonisation
1615 regardless of the development of phage resistance amongst the target
1616 population. Such trade-offs occur due to maladaptive pleiotropic effects whereby
1617 the enhancement of one component of bacterial fitness compromises another
1618 (Lenski, 1988). As such, the evolution of phage resistance, which enhances
1619 bacterial fitness through the ability to evade the bactericidal activity of lytic phage,
1620 can result in a trade-off with other bacterial traits such as their competitive ability
1621 (Mangalea and Duerkop, 2020). For example, one mechanism by which bacteria
1622 acquire resistance is through the loss or modification of receptor molecules that
1623 are used by phage to bind and initiate infection (Majkowska-Skrobek et al., 2021).
1624 Due to the shared role of these receptors in the uptake of nutrients, phage
1625 resistance results in the simultaneous reduction in competitive ability (Stone et
1626 al., 2019). Although the primary aim of treatment with phage continues to be the
1627 clearance of pathogenic bacteria, the combined use of probiotics means that the
1628 evolution of resistance does not indicate the failure of therapy, but an opportunity
1629 to initiate a secondary attack that capitalises on the compromised competitive
1630 ability of the remaining resistant bacteria. This concept is sometimes described
1631 as phage steering, defined as the use of phage 'to kill bacteria but also steer
1632 survivors towards resistant but more compromised phenotypes' (Gurney et al.,
1633 2020). Our strategy not only relies on the existence of trade-offs but also on
1634 frequency dependent selection. The killing action of phage acts to reduce the
1635 frequency of the target bacterium which increases the competitive advantage of
1636 the probiotic by allowing it to outnumber its competitor. This competitive

advantage is a result of the influence of density on the outcome of ecological competition where even a stronger competitor can be prevented from colonising by a weaker competitor if that weaker competitor is present at high frequencies (Narise, 1965). Ultimately the effect of phage and probiotic complement one another to provide a novel antimicrobial strategy that buffers against the evolution of resistance through the artificial manipulation of evolutionary and ecological interactions.

The primary objective of this study is to explore the understudied concept of the use of phages as probiotics to reduce the persistence of multi-drug resistant lineages in the gastrointestinal tract. More specifically we explore whether the dual application of lineage-specific phage alongside antagonistically strong probiotic competitors can increase the likelihood of decolonisation success. Principally we will investigate the likelihood of phage resistance evolving and attempt to characterise the rate at which this occurs. We will also use a series of competition experiments to test the hypothesis that exposure to phage will reduce the competitive fitness of resistant genotypes relative to a standard enteric competitor (Nissle). We will investigate factors that may alter bacterial susceptibly to phage such as whether the evolution of resistance can be slowed via the use of phage cocktails. To determine the most effective combination of phages to use, phage susceptibility tests will be carried out to identify phage that target distinct resistance mechanisms possessed by the target bacterium. In doing so we aim to reduce the likelihood of complete resistance emerging as this would require the simultaneous evolution of multiple resistance mechanisms (Yang et al., 2020). The ability to reduce the prevalence of resistant genotypes through altering the competition between strains may enable us to reduce the relative fitness of resistant genotypes compared to susceptible ones. If successful, this

1663 two-tiered antimicrobial strategy will provide us with a method with which we
1664 could selectively remove resistant bacteria from the population and therefore
1665 increase susceptibility to antibiotics. Demonstration of the ability of this strategy
1666 to overcome the evolution of resistance would provide incredibly compelling
1667 support for the widespread implementation and uptake of phage therapy in
1668 clinical settings and would validate the contribution of applying an evolutionary
1669 perspective to the exploration and resolution of public health problems.

# Materials and Methods

## Competition experiment

To assess the effect of phage predation and ecological competition on the fitness of ST131, two 7-day competition experiments were carried out. For the first 7-days, four treatments were set up, each represented by six replicates.

Competition treatments: ST131 only (Control); ST131 and Nissle (NIS); ST131 and Phage H alone (φ); ST131, Phage H and Nissle (φ + NIS).

A 50:50 starting ratio of ST131 and Nissle was achieved by diluting overnight cultures 1:100 into LB broth and phage was added at a multiplicity of infection (MOI) of 0.1. All treatments used 6ml of LB broth and were incubated in a shaking incubator (180 rpm) at 37 $^{\circ C}$ for 24-hour periods.

Phage H was isolated from pig faeces using ST131 isolate EC958 as part of a previous study (Barron, 2019). It had been confirmed to successfully infect EC958 but not *E. coli* Nissle (Barron, 2019). Using the same methodology described in Chapter 1, Illumina sequencing identified phage H as a member of the family Autographiviridae with an estimated taxonomic characterisation of *E. coli* virus LL11.

At the onset of the experiment (T0), a 200μl sample was taken from each replicate of all four treatments and used to calculate CFU/mL and PFU/mL.

## Calculation of CFU/mL

100µl of each sample was used to conduct 10-fold serial dilutions in M9 buffer. Plating of several dilutions was carried out per sample to ensure that at least 1-2 plates were within a suitable colony counting range (approximately 25-250 colonies per plate).

Each replicate was plated on ChromoSelect Agar (Merck, Darmstadt) and on LB agar containing cefotaxime (5µg/ml). When cultured on ChromoSelect Agar, ST131 and Nissle colonies show up as distinct colours (purple and blue respectively, Figure 1) providing a reliable method for the visual detection and differentiation of both strains on the same plate. As ST131 is cefotaxime-resistant, plating on LB containing cefotaxime can also be used, and is a more traditional method, for distinguishing between MDR ST131 and susceptible Nissle colonies through the comparison of the number of colonies on antibiotic free plates. All plates were incubated overnight at 37°C and colonies were counted the following day. This process was repeated for each replicate of all treatments every 24 hours over a 7-day period.

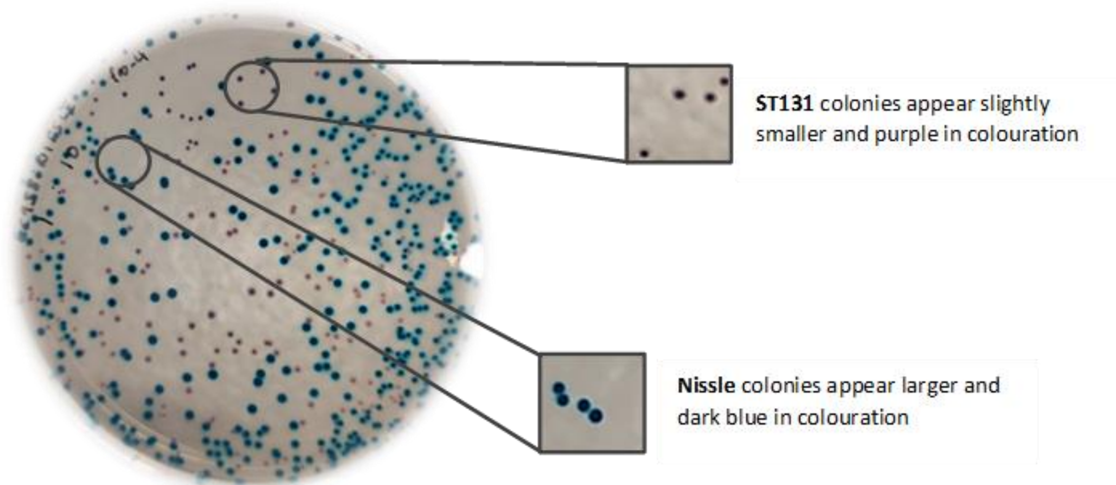



**Figure 1.** Colour difference between *E. coli* ST131 colonies and *E. coli* Nissle colonies when grown on ChromoSelect Agar

## Calculation of PFU/mL

Chloroform was added to 100µl of sample at a ratio of 1:10. Samples were centrifuged for 25 minutes at 3500 rpm, at 4$^{\circ C}$ and then the supernatant removed. 10-fold serial dilutions were conducted in M9 buffer and then spotted (5µl) on overlay plates of EC958 (15ml 0.5% soft agar, 300µl EC958). T0 is the only timepoint at which this process was conducted for the two control treatments to ensure no initial phage contamination. At all proceeding timepoints, PFU/mL was only recorded for the two experimental treatments.

At each 24-hour period, 60µl from each rep was transferred into 6ml of fresh LB broth to refresh depleted nutrients. 50µl was also taken in order to make glycerol stocks (30%) that were stored at -80$^{\circ C}$.

## Extension of the 7-day competition

To investigate the longer-term trajectory of bacterial and phage dynamics, the experiment was set up to run for a further 7-days using glycerol stocks taken from the final timepoint of the previous experiment. Both phage containing treatments were divided in two to allow us to investigate the effect of the addition of a further volume of phage (added at a MOI of 0.1);

φ (+ φ) – Additional supplement of phage H at onset of second 7 days

φ + NIS (+ φ) – Additional supplement of phage H at onset of second 7 days

From T7 onwards, treatments φ and φ + NIS are represented in all analysis and figures by the above.

PFU/mL and CFU/mL were measured at T8, T11 and T14 and no further resistance assays were conducted.

## Detection of the emergence of phage resistance

Phage resistance assays were conducted at two timepoints during the experiment (T4 and T7) to determine any change in the sensitivity of ST131 to phage H. For each replicate of all treatments, 48 individual colonies were picked using a cocktail stick from LB agar plates containing cefotaxime to ensure the colonies picked were ST131. Each colony was placed into the well of a 96-well plate containing 200µl of LB medium and placed in a shaking incubator (180 rpm) at 37℃ overnight. The following day, each colony was transferred into a well containing M9 buffer to achieve a 10-fold dilution. 25µl of a 10x dilution of phage H (in M9 buffer) was used to prepare six virus lines per square LB agar plate.

1747 Once dry, 12 colonies were streaked through each virus line and incubated
1748 overnight at 37℃. WT EC958 and Nissle were also streaked as controls.
1749 Resistance is indicated by continued bacterial growth across the virus line
1750 whereas susceptibility is indicated by a reduction in bacterial growth across the
1751 virus line.

1752

## 1753 Phage host range testing for phage cocktail

1754

1755 To identify if the mechanisms that confer resistance to phage H provide cross
1756 resistance to other phages, a series of host range tests were carried out.

1757 Our phage library was assessed and a total of 18 phages identified as capable of
1758 infecting EC958 were amplified. Spot assays of all phages were conducted on
1759 both WT EC958 and Nissle and any that were able to infect Nissle were
1760 discarded. Spot assays were then carried out on overlay plates (15ml 0.5% soft
1761 agar, 300µl of one of 24 phage H resistant EC958 colonies isolated from
1762 competition experiments) using the remaining phages, as well as phage H as a
1763 control. As a control, spot assays were also performed on 4 susceptible colonies
1764 as well as on WT EC958 and Nissle. PFU/mL was calculated per phage on each
1765 strain to determine susceptibility of the strain to phage infection.

1766

## 1767 Measuring the fitness cost of phage resistance

1768

1769 To identify any fitness costs associated with phage resistance, competitions
1770 between phage resistant EC958 colonies and Nissle were conducted alongside
1771 phage susceptible (WT) EC958 and Nissle. Due to known frequency dependent

1772 effects of higher starting frequency among these two strains, initial densities of
1773 both ST131 and Nissle needed to be as close to 50:50 as possible. To achieve
1774 this, a culture of each strain was grown overnight in a shaking incubator at 37$^{\circ C}$.
1775 The optical density of each culture was then measured and used to standardise
1776 the optical density of each strain in fresh 6ml vials of LB broth. These were then
1777 grown overnight under the same conditions and optical density measured again
1778 the following morning. These measurements were used to determine the ratio of
1779 each strain that was used to create each master mix. An independent master mix
1780 was made up for each strain used. A total of three phage resistant colonies were
1781 used and are referred to as;

1782 Res 1 – resistant colony taken from T3 of φ treatment

1783 Res 2 – resistant colony taken from T7 of φ treatment

1784 Res 3 – resistant colony taken from φ + NIS treatment

1785

1786 6 replicates of each experimental treatment and 12 replicates of the control
1787 treatment were set up using 60µl of the corresponding master mix. These were
1788 then grown over a period of 24 hours in a shaking incubator (180 rpm) at 37$^{\circ C}$ .

1789

1790 The four treatments were as follows;

1791 Res 1 + NIS

1792 Res 2 + NIS

1793 Res 3 + NIS

1794 WT EC958 + NIS (control)

1795 A 200µl sample was taken from each master mix and used to conduct serial
1796 dilutions in M9 buffer and plated out on LB agar and LB agar containing
1797 cefotaxime (8mg/mL) to provide an indication of the initial ratios of both strains
1798 present in the competition.

1799 After 24 hours, the same process of plating out was conducted and the counts
1800 achieved the following day were used to calculate final ratios of ST131:Nissle in
1801 each treatment. Initial and final ratios were then used to calculate the relative
1802 fitness of all three resistant isolates when in competition with Nissle using the
1803 following formula (Ross-Gillespie et al., 2007);

1804

1805 Relative fitness = [(fraction of ST131 at T1) * (1 – (fraction of ST131 at T0))] /
1806 [(fraction of ST131 at T0) * (1 – (fraction of ST131 at T1)]

1807

1808 Distinct morphological differences were identified between all three resistant
1809 colonies of EC958 and Nissle when grown on the same LB agar plate without
1810 cefotaxime (Figure 2). The plaques of the resistant colonies when grown on
1811 plates containing cefotaxime were tiny and opaque making for inaccurate count
1812 data. We therefore decided to use the counts from the same plate. As WT EC958
1813 and Nissle do not differ strongly enough in their morphology to be able to gain an
1814 accurate count from the same plate, and due to time constraints, our control data
1815 has been taken from a previous experiment conducted by Barron (2019).

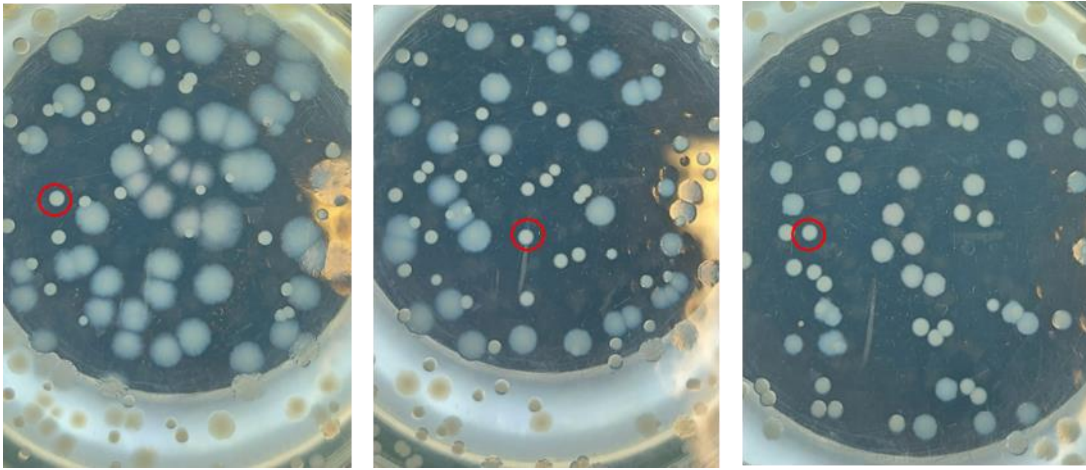


**Figure 2.** Morphological differences between phage H resistant colonies of EC958 and Nissle when grown on LB agar. From left to right, RES 1, RES 2 and RES 3. Examples of the morphologies of resistant colonies are circled in red. Nissle colonies were larger and less spherical.

All data analysis was conducted in RStudio 1.1.442.

# Results

## The greatest reduction in the density of ST131 was achieved using phage and Nissle combined

To test the effect of the presence of Nissle and phage H on the density of ST131, we conducted a linear mixed model with the inclusion of quadratic, cubic and interaction terms using the lmer function in the lme4 package (Bates et al., 2015). We used a model simplification method to determine the significance of our main effects. Nissle had a significant effect on the density of ST131 (Figure 3A, Likelihood ratio test Nissle versus no Nissle, df=1, $\chi^2$=44.3, P < 0.0001) as did phage H (Figure 3A, Likelihood ratio test Phage versus no Phage, df=1, $\chi^2$= 24, P < 0.0001). The effects of phage on reducing ST131 density were greatest when Nissle was present (Figure 3A, Likelihood ratio test PhageXcompetition df = 1, $\chi^2$= 48.0, P < 0.0001). We confirmed that our minimum adequate model possessed the lowest AIC of 576 with further model simplification leading to an increase in AIC.

## The population dynamics of Nissle are unaffected by the presence of phage

To determine if the addition of phage had any impact on the density of Nissle we conducted a linear mixed model with the inclusion of quadratic and cubic terms using the lmer function in the lme4 package (Bates et al., 2015). We used a model simplification method and were able to conclude that the addition of phage had no significant impact on the density of Nissle (Figure 3B, Likelihood ratio test

Phage versus no Phage, df=1, $\chi^2$= 0.2, P < 0.05). Our minimum adequate model had the lowest AIC value of 301.

## The presence of Nissle has a significant effect on the density of phage H

To determine whether the presence of Nissle had any impact on the density of Phage H we conducted a linear mixed model using the lmer function in the lme4 package (Bates et al., 2015). We used model simplification to confirm that the presence of Nissle had a significant effect on the $log_{10}$ transformed density of phage (Figure 3C, Likelihood ratio test Nissle versus no Nissle, df=1, $\chi^2$=8.1, P < 0.01). Our minimum adequate model containing the Nissle treatment was confirmed to have the lowest AIC value of 369.

## Second addition of phage had no impact on dynamics

I used t-tests to determine whether the addition of phage at the onset of the second 7 days of competition had a significant effect on the CFU/mL and PFU/mL counts obtained for treatments φ and φ + NIS. The addition of phage had no significant effect on the CFU/mL of ST131 in either treatment (Two sample t-test, φ versus φ (+ φ), t=-0.018, df=34, P > 0.05; φ + NIS versus φ + NIS (+φ), t=-1.15, df=34, P > 0.05). The addition of phage had no significant effect on the CFU/mL of Nissle (Two sample t-test, φ + NIS versus φ + NIS (+φ), t=-0.73, df=34, P > 0.05). The addition of phage did have a significant effect on the PFU/mL of phage H in both treatments (Two sample t-test, φ versus φ (+ φ), t=-3, df=34, P < 0.05; φ + NIS versus φ + NIS (+φ), t=-3.83, df=34, P < 0.05). All φ and φ + NIS

1869 treatments are therefore represented in all analysis and figures from T7 onwards

1870 by the treatments that are exposed to additional phage.

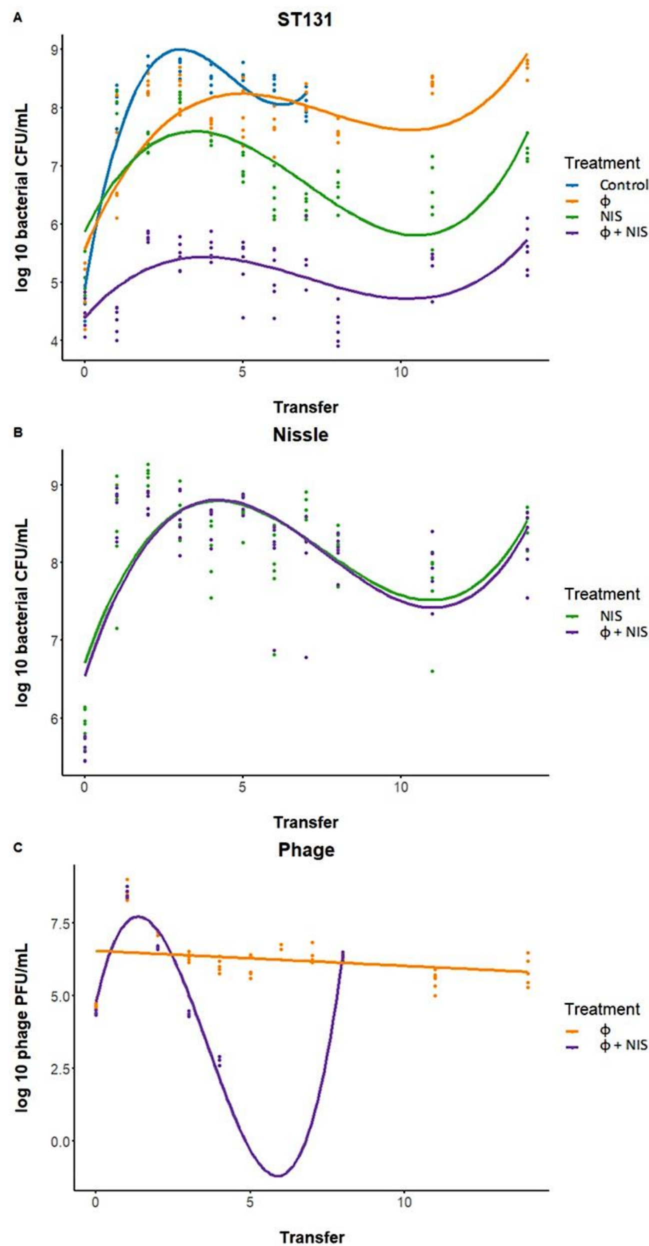



**Figure 3.** Population dynamics of ST131, Nissle and phage H over the course of a 14-day competition experiment. **3A** shows how the log10 density of ST131 changes when in competition with Nissle (NIS), when exposed to phage H (φ) and when exposed to Nissle and phage H simultaneously (φ + NIS). For phage containing treatments, additional phage was added at T8. ST only control was only run for the first 7 days of the experiment. Polynomial regressions plotted. **3B** shows how the log10 density of Nissle changes when in competition with ST131 (NIS) and when in competition with ST131 in the presence of phage H (φ + NIS). Polynomial regressions plotted. **3C** shows how the log10 density of phage H changes when in competition with ST131 only (φ) and when also in the presence of Nissle (φ + NIS). Linear regression plotted for treatment (φ) and polynomial regression for treatment (φ + NIS).

1871 **Comparison of the fraction and relative fitness of ST131**
1872 **between competition only (NIS) and competition plus phage (φ**
1873 **+ NIS) treatments**

1874

1875 In order to determine whether the fraction of ST131 from T2 to T14 is significantly
1876 different between our competition only (NIS) treatment and our combination
1877 treatment (φ + NIS) we conducted a linear mixed model. We used a model
1878 simplification method which revealed that the fraction of ST131 was significantly
1879 different between treatments (Figure 4A, Likelihood ratio test, Phage versus no
1880 phage, df=1, $\chi^2$= 36.7, P < 0.00001).

1881 We also used model simplification to investigate whether the significant
1882 difference in the fraction of ST13 between treatments was stable across transfers.
1883 Data from T0-T1 was excluded from this analysis as during this time variation in
1884 the fraction of ST131 can be attributed to attempts to reach an equilibrium.
1885 Transfer had no effect on the fraction of ST131 (Figure 4A, Likelihood ratio test,
1886 Transfer versus no transfer, df=1, $\chi^2$=0.1, P = 0.75). Our minimum adequate
1887 model had the lowest AIC of 222 and linear regressions are plotted.

1888 We also carried out direct comparisons of the relative fitness of ST131 between
1889 treatments NIS and φ + NIS for multiple timepoints throughout the experiment.
1890 There was a significant difference in the relative fitness of ST131 between
1891 treatments between T0-T1 (ANOVA, $F_{1,8}$=111.5, df= 1, P < 0.0001), T0-T2
1892 (ANOVA, $F_{1,9}$=34.4, df=1, P < 0.001), T1-T2 (ANOVA, $F_{1,9}$=60.2, df=1, P <
1893 0.0001) and T0-T14 (ANOVA, $F_{1,9}$=34.3, df=1, P < 0.001).

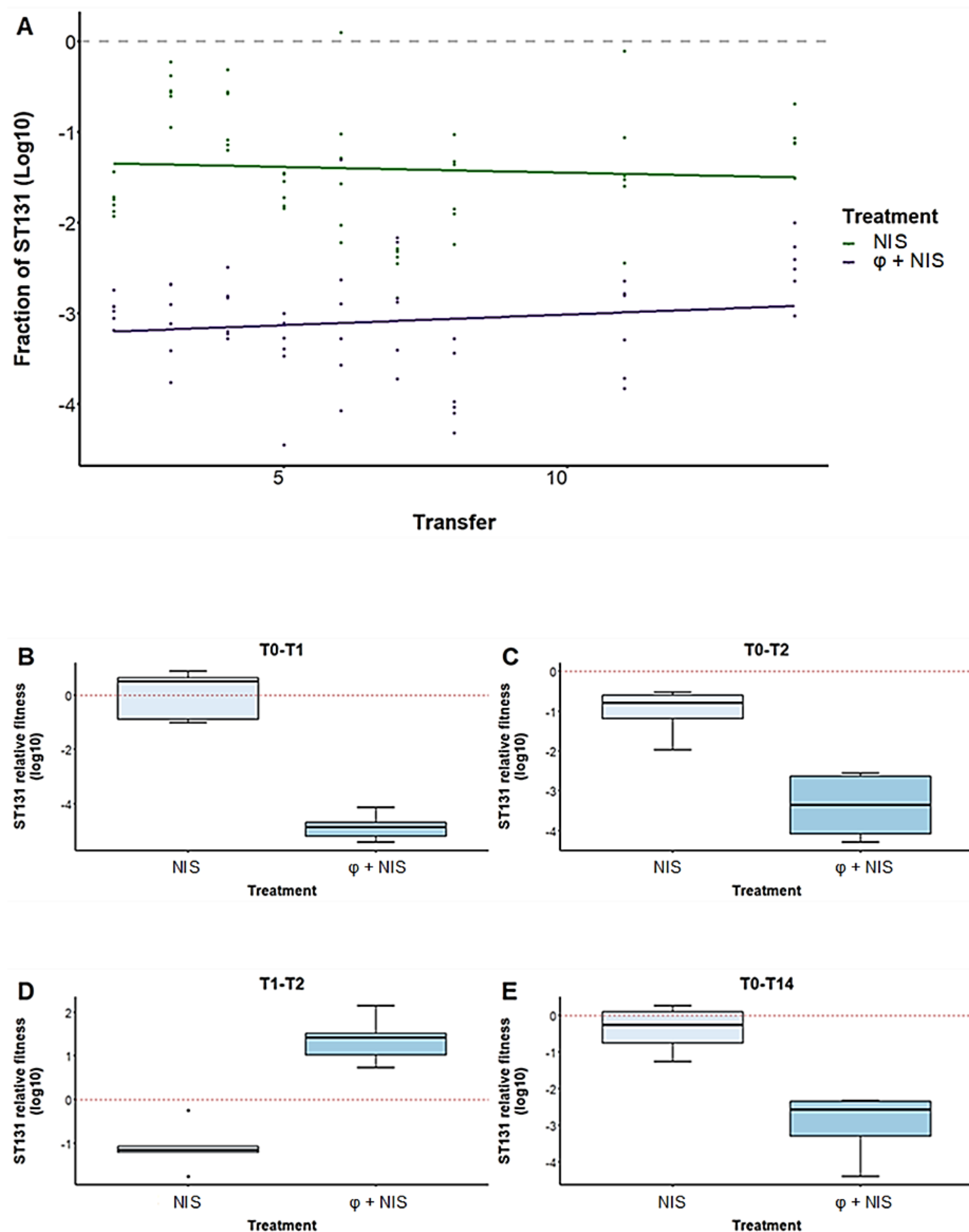



**Figure 4.** Comparison of the relative fitness of ST131 (Log10) between treatments NIS and φ + NIS over the course of a 14-day competition experiment. **4A** shows the fraction of ST131 between T2-T14 when in competition with Nissle with and without the addition of phage. Linear regression is displayed. A relative fitness below dashed line at 0 indicates that ST131 has a lower competitive ability than Nissle. **4B** displays boxplots representing the relative fitness of ST131 between treatments at four distinct timepoint comparisons throughout the experiment. Relative fitness above the red dashed line at 0 represents a higher relative fitness.

1894 # The evolution and costs of phage resistance

1895

1896 **1.1 The presence of Nissle did not slow the rate at which phage resistance**

1897 **evolved.**

1898 To investigate whether resistance was less likely to evolve or would evolve at a
1899 slower rate when exposed to phage and Nissle combined, resistance assays
1900 were conducted at T3 and T7 for treatment φ and φ + NIS . We found no
1901 significant difference between the level of resistance between treatments at either
1902 T3 or T7 (Figure 5A, Two Sample t-test, t=0.85, df=22, $P > 0.05$).

1903

1904 **1.2 The evolution of phage resistance is not costly with resistant mutants**

1905 **possessing higher relative fitness than their WT counterparts.**

1906 To determine whether phage resistance resulted in a reduction in the relative
1907 fitness of ST131 when in competition with Nissle, we compared the relative
1908 fitness of WT ST131 to three phage resistant colonies isolated from resistance
1909 assays. A one-way ANOVA was revealed that there was a significant difference
1910 in the relative fitness of WT and resistant ST131 with resistant ST131 possessing
1911 higher relative fitness compared to its WT counterpart (one-way ANOVA,
1912 $F_{3,30}=6.34$, $P < 0.01$).

1913 To test whether this difference was present between WT ST131 and all resistant
1914 colonies we conducted a generalised linear model (GLM) which revealed that
1915 although the relative fitness of RES1 and RES2 was significantly different from
1916 that of our WT ST131, the relative fitness of RES3 was not (RES1, GLM, t=4.02,
1917 $P < 0.001$; RES2, GLM, t= 2.61, $P < 0.01$; RES3, GLM, t=0,96, $P > 0.05$).

**A**

1918

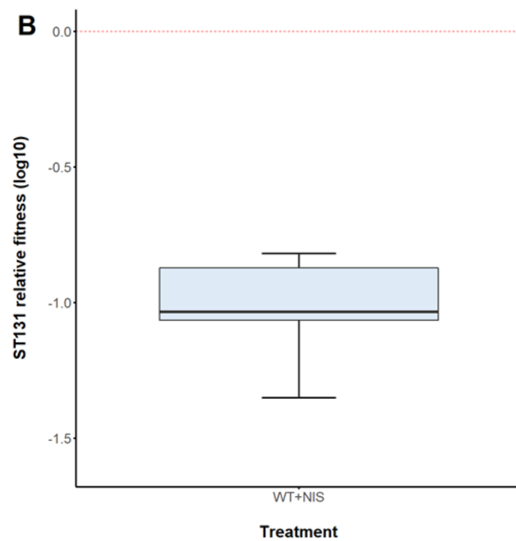
| | Timepoint of Resistance Assay | |
|---|---|---|
| **Treatment** | **T3** | **T7** |
| ϕ | 0.99 | 1 |
| ϕ + NIS | 1 | 0.98 |

1919

1920

1921

1922



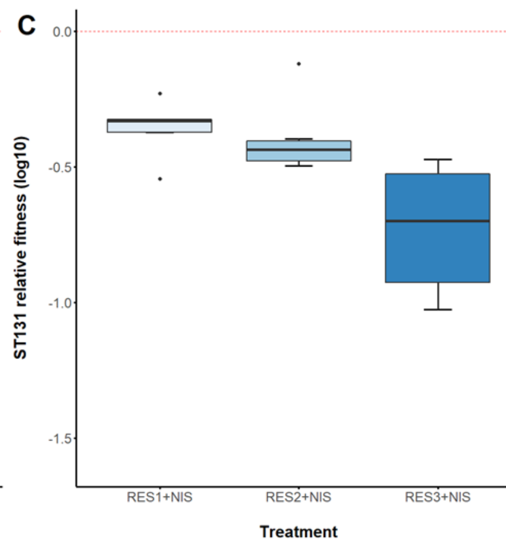




**Figure 5.** Prevalence of phage resistance and variation in the relative fitness of WT and resistant ST131 colonies when in competition with Nissle. **5A** Proportion of phage resistant ST131 colonies at timepoint 3 and timepoint 7 in the presence (φ + NIS) or absence (φ) of Nissle. Results obtained from resistance assays. For each treatment approximately 40-48 colonies per rep (6 reps per treatment) were assayed. **5B** The relative fitness of WT ST131 when competed with Nissle. This data was extracted from a previous, unpublished, study by Barron (2019). **5C** The relative fitness of three independent phage resistant ST131 colonies extracted from resistance assays conducted on day 3 and 7. RES1 and RES2 are colonies taken from φ treatments at T3 and T7 respectively and RES3 was a colony isolated from φ + NIS treatment at T7. For boxplots in 5B and 5C, whiskers represent the first and third quartile of the data with the median represented by the central line. Relative fitness below 0 indicates that ST131 was outcompeted by Nissle. Caution must be taken when interpreting the results of these tests as the fitness measurements of our WT ST131 and resistant colonies were taken from independent investigations.

1929 **Identification of cross-resistance to inform phage cocktail**
1930 **development**

1931

1932 To determine whether resistance to phage H also provided ST131 with resistance
1933 to other ST131-specific phage we isolated 24 colonies from our 14-day
1934 competition experiment that had been identified through resistance assays as
1935 phage resistant. These were screened against 10 phage isolated as part of this
1936 study including phage H. All 24 resistant colonies were susceptible to all phages
1937 except phage H. We also screened 4 phage sensitive colonies as well as as WT
1938 ST131 and Nissle to ensure that the spot assay method used would provide an
1939 accurate method for measuring resistance. All four sensitive colonies were
1940 infected by all 10 phage, as was WT ST131. Nissle was not susceptible to any of
1941 the phage confirming that the phage used are ST131-specific.

**Table 1.** The mean PFU/mL of 10 ST131-specific phages on 24 phage resistant colonies, 2 phage sensitive colonies, WT ST131 and Nissle.

| Phage I.D. | Mean PFU/mL Resistant colonies | Mean PFU/mL Sensitive colonies | Mean PFU/mL WT ST131 | Mean PFU/mL Nissle |
|---|---|---|---|---|
| **FalwwA1** | 372500 | 600000 | 280000 | N/A |
| **FalwwB1** | 506666.6667 | 640000 | 500000 | N/A |
| **FalwwC1** | 369166.6667 | 325000 | 340000 | N/A |
| **HayA1** | 4516666667 | 3E+09 | 320000000 | N/A |
| **HayB1** | 13466666667 | 1.6E+10 | 1800000000 | N/A |
| **HayC1** | 1.37125E+64 | 1.3E+10 | 1800000000 | N/A |
| **Phage H** | N/A | 6.75E+08 | 140000000 | N/A |
| **Phage G** | 14608695652 | 3.1E+09 | 4200000000 | N/A |
| **GorA7** | 26097252.17 | 14500 | 5600000000 | N/A |
| **GorC7** | 191672950 | 7050 | 3000000000 | N/A |

1942 # Discussion
1943

1944 The aim of this study was to determine the efficacy of the combined use of
1945 phage and probiotic *E. coli* for the eradication of MDR ST131. If successful, this
1946 approach is intended be applied *in vivo* to achieve the intestinal decolonisation of
1947 ST131 and, subsequently, reduce the risk of developing future extra-intestinal
1948 infections (Pires et al., 2016). The complimentary use of probiotic bacteria to
1949 improve the therapeutic outcome of the use of phage is an example of adjunctive
1950 therapy (Ganeshan and Hosseinidoust, 2019). Although there are many
1951 examples of the use of probiotics and phage as adjuncts to antibiotics, there is
1952 little evidence of their use to enhance the efficacy of one another (Spinler et al.,
1953 2017; Tkhilaishvili, et al., 2019). Our two-tiered strategy is centred on the notion
1954 that ST131-specific phage will successfully reduce the frequency of ST131 to a
1955 level that can be driven to extinction through competition with the antagonistically
1956 strong probiotic strain, *E. coli* Nissle 1917. The presence of Nissle can buffer
1957 against the diminishing effectiveness of phage predation that results from the
1958 evolution of phage resistance. In return, the reduction in the frequency of the
1959 target bacterium achieved by phage acts to increase the competitive advantage
1960 of Nissle via positive frequency dependent selection.

1961 The results of our study indicate that the concurrent use of phage and probiotics
1962 presents a promising therapeutic strategy for the targeted removal of multidrug
1963 resistant pathogens. Figure 3A demonstrates the effectiveness of four different
1964 treatments in decreasing the CFU/mL of ST131 over the course of a 14-day
1965 experimental period. Although the independent application of both Nissle and
1966 phage H resulted in a significant decrease in the density of ST131, the reduction
1967 achieved by phage H was the weakest, likely due to the evolution of phage

resistance by T3 (Figure 5). As a result, the dynamics observed for the remainder of the experiment in this treatment group closely mimic those seen in our ST131-only control. In contrast, the use of our probiotic competitor, Nissle, resulted in a reduction in ST131 of over two orders of magnitude (Figure 3A). However, the combined application of phage H and Nissle resulted in a significantly greater reduction in ST131 density than that achieved by either phage or Nissle alone (Figure 3A). This provides compelling support for our proposed synergistic use of phage and probiotic strains to achieve the intestinal decolonisation of *E. coli* ST131. Research into the synergetic combination of antimicrobial agents is still largely dominated by examples of the use of alternative antimicrobials to improve the efficacy of antibiotics (Liu et al., 2020). Turning our attention to the potential of other, more unusual, combinations of antimicrobials, such as probiotics and phages will enable us to reduce our reliance on antibiotics and expand the diversity of our therapeutic arsenal against multi-drug resistant pathogens (Annunziato, 2019).

Although our approach was successful in reducing the frequency of ST131, it failed to eradicate it from the population. Figure 4 shows how the fraction of ST131 changes over the course of the experiment. After experiencing an initial rapid decline from T0-T1, the frequency of ST131 in our combination treatment bounces back and reaches a relatively stable equilibrium of approximately 0.001% of the total population. The increase in the frequency of ST131 from T1-T2 can be explained by the evolution of phage resistance which provides ST131 with the opportunity to recover from the initial decline in frequency. The seemingly stable persistence of ST131 at such a low frequency in the population is in contrast with our initial hypothesis which predicted the extinction of ST131 as a result of positive frequency dependence. Frequency dependent selection occurs

when ‘the fitness of a genotype or phenotype depends on its frequency within the population’ (Lande, 1976; Svensson and Connallon, 2019). We hypothesised that as the proportion of ST131 in the population is driven down by phage predation, the proportion of Nissle will increase. The increased frequency of Nissle in the population would act to increase its fitness which would in turn accelerate the rate of extinction of ST131. This hypothesis was formulated based on the knowledge that positive frequency dependence is a classic feature of competition between two microbes of the same species as well as the reported ability of Nissle to outcompete strains of ST131, and other species, when competed at equal, as well as unfavourable, initial frequencies (Barron, 2019; Hancock, Dahl and Klemm, 2010). Commonly, the conclusions of such studies are based on the extrapolation of data collected from competition assays conducted over a 24-hour period and fail to consider how the population dynamics of the target population may change following this period. For example, in the first 24 hours of our experiment, we observe a rapid decline in the frequency of ST131 which, supported by the negative relative fitness of ST131, would indicate a global trend towards the extinction of ST131 from the population (Figure 3A). However, by tracking bacterial dynamics for a period of 14 days, we show that this is not the case, with ST131 able to remain in the population at a relatively low, yet stable, frequency. This highlights the unreliability of predicting the outcome of microbial interactions from short-term evolution experiments and raises questions as to the usefulness of our two-tiered strategy as an agent for intestinal decolonisation. Though the ability of our two-tiered approach in achieving significant reductions in the frequency of ST131 is encouraging, complete decolonisation is required if we are to effectively reduce the risk of patients developing spontaneous extraintestinal infections and the high levels of mortality associated with them.

2020 Investigation into the factors responsible for the persistence of ST131 in vitro will
2021 allow us to assess whether our two-tiered approach will be capable, with some
2022 modifications, of achieving this.

2023 The conditional expression of anti-competitor toxins may provide one
2024 explanation for the stable, low frequency persistence of ST131 in our
2025 experimental populations (Bhattacharya, Pak and Bashey, 2018). One method
2026 that bacteria employ to compete for resources and space is the production of
2027 bacteriocins, proteinaceous antibacterial toxins that are principally active against
2028 closely related strains (Ghazaryan, Giladi and Gillor, 2019; Soltani et al., 2020).
2029 Bacteriocin production is a prime example of interference-based competition,
2030 typically characterised by the use of antagonistic factors to eliminate competitors
2031 from the environment (Paquette et al., 2018; Stubbendieck, Vargas-Bautista and
2032 Straight, 2016). The production and secretion of bacteriocins is metabolically
2033 expensive, as evidenced by the reduced growth rate of toxin-producing strains
2034 compared to their non-producing counterparts and represents a classical
2035 'example of spiteful behaviour that is expensive for the producer and harmful for
2036 others' (Bucci, Nadell and Xavier, 2011; Doekes, de Boer and Hermsen, 2019).
2037 *E. coli* are known to produce two main types of bacteriocin categorised by their
2038 molecular weight into colicins and microcins (Budic et al., 2011). Although both
2039 are activated in times of stress, their method of secretion differs, with cell lysis
2040 required to achieve the release of colicins (Ghazaryan et al., 2014; Budic et al.,
2041 2011; Inglis et al., 2013; Bayramoglu et al., 2017). Interestingly, the production of
2042 bacteriocins, specifically microcin M and H47, has been reported as central to the
2043 success of probiotic strain *E. coli* Nissle (Nissle) in microbial competitions
2044 (Sassone-Corsi et al., 2016; Pradhan et al., 2020). The high metabolic costs
2045 associated with toxin production means that some form of regulatory system must

2046 be in place to allows strains to adjust their energetic investment in bacteriocin
2047 production based on the changing benefit it provides over the course of
2048 competition. Examples of such competitor dependent production of bacteriocins
2049 has been evidenced in other species such as *Lactobacillus plantarum*
2050 (Maldonado-Barragán et al., 2013). This regulation allows strains to avoid paying
2051 the metabolic cost of production when the benefits of employing bacteriocins are
2052 low (Maldonado-Barragán and West, 2020). The maintenance of ST131 in the
2053 population at a proportion of approximately 0.001% suggests that there is a
2054 fitness benefit of it remaining rare and is indicative of the influence of negative
2055 frequency dependent selection. We hypothesize that the conditional regulation of
2056 Nissle's bacteriocin production on the presence of a competitor could explain this
2057 phenomenon. The level of bacteriocin production favoured by natural selection is
2058 not fixed but varies based on two key aspects of a strain's social environment:
2059 the level of relatedness, or kinship, between members of the population and the
2060 scale of competition (Gardner and West, 2004). As the killing action of
2061 bacteriocins alters the population structure, the relatedness among strains and
2062 scale of competition changes, resulting in a shift in the direction of selection on
2063 bacteriocin production. Production is favoured when producing cells are at
2064 intermediate starting frequencies in the population and the scale of competition,
2065 which may be inferred through competition sensing from the degree of cellular
2066 damage or stress, is high (Inglis et al., 2012). Both conditions are met at the onset
2067 of our competition experiments where the starting frequency of Nissle and ST131
2068 is 50%. As the frequency of ST131 is driven down by Nissle, the benefit of
2069 producing bacteriocins is reduced due to a higher level of relatedness among
2070 neighbouring cells as well as a lower level of competition from the increasingly
2071 shrinking size of the ST131 population. Under these conditions, the costs of

2072 bacteriocin production outweigh the benefits and downregulation is favoured. As
2073 this downregulation occurs prior to the extinction of ST131, a small proportion are
2074 able to remain in coexistence with Nissle. The stability of this coexistence
2075 appears to be maintained by the threat of bacteriocin upregulation. If the
2076 proportion of ST131 was to increase and the level of relatedness and competition
2077 within the population tipped the balance in favour of bacteriocin production,
2078 ST131 would once again be exposed to toxin attack. To avoid this toxin
2079 'kickback', we propose that ST131 persists at a low frequency in the population
2080 which allows it to escape the antagonistic activity of its competitor. It is important
2081 to consider that the relative costs and benefits of bacteriocin production are
2082 largely determined by a strain's social environment. Although in our experiment,
2083 this consists of just one conspecific, *in vivo* the interaction between Nissle and
2084 ST131 will take place within the complex social community of the gut. It will be
2085 crucial to conduct these experiments in conditions more representative of this
2086 environment such as through the use of organoids or anaerobic chambers to
2087 determine whether the same population dynamics can be observed in more
2088 structured and diverse environments. Additionally, conducting these experiments
2089 using spent media would allow us to track the production of bacteriocins over the
2090 course of competition and provide data that may support or disprove this
2091 interesting biological explanation for the low-level persistence of ST131.

2092 Cellular heterogeneity may provide an alternative explanation for the
2093 persistence of ST131 in our experimental population (Altschuler and Wu, 2010;
2094 Huang, 2009; Waldherr S., 2018). Population-averaging methods are commonly
2095 used to analyse the behaviour of cellular populations but fail to recognise that,
2096 despite their genetic clonality, bacterial populations are rarely phenotypically
2097 homogeneous (Kester and Fortune, 2014). In fact, a degree of individuality

2098 among cells, or subpopulations of cells, is present to some degree within any
2099 population and can have functional significance (Altschuler and Wu, 2010). A
2100 prominent example of this is in the development of cancer, a disease that is driven
2101 by cellular heterogeneity in growth rate. In the case of cancer, the cause of such
2102 variation is most commonly genetic, but many studies have shown that
2103 phenotypic variation is also responsible for the development of distinct
2104 subpopulations (Kester and Fortune, 2014). Arguably, the existence of these
2105 small and phenotypically distinct subpopulations able to persist in a range of
2106 environmental conditions may be seen to act as a bet-hedging mechanism that
2107 enables populations to mitigate the risk of extinction in unpredictable
2108 environments (Jolly et al., 2018). For example, several studies have
2109 demonstrated that in the face of antibiotic administration, a small subset of cells
2110 are able to adjust their physiological state and switch to what is known as a
2111 persister phenotype (Wood, Knabel and Kwan, 2013). These persisters exist in
2112 an arrested state of growth that enables them to survive antibiotic exposure.
2113 Evidence of persister cells has been found across many bacterial species
2114 including *E. coli*, *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas*
2115 *aeruginosa (P. aeruginosa*). This phenotypic switching can occur not just as a
2116 response to antibiotic exposure but to other environmental stressors including an
2117 increase in bacterial cell densities (Vulin, et al., 2018). Persistence represents the
2118 most extreme form of tolerance, whereby cells are metabolically inactive, or
2119 dormant (Trastoy, et al., 2018). However, such rigid categorisation of cells as
2120 either metabolically active or inactive (persisters) is likely to mask much of the
2121 intermediary variation present within populations (Huang, 2009). A subset of cells
2122 all categorised as metabolically inactive may in fact exhibit a range of metabolic
2123 activity, with cells able to tolerate and survive stressors in their environment, not

2124 just via complete growth arrest, but through the slowing down of other essential
2125 bacterial processes (Trastoy et al., 2018; Westblade, Errington and Dörr, 2020).
2126 It is plausible that the 0.001% of ST131 remaining at the endpoint of our
2127 competition assays is evidence of a phenotypically distinct subpopulation of
2128 ST131 able to tolerate the selective pressures imposed by the presence of phage
2129 and conspecific competitor, Nissle. To determine whether this is the case, we
2130 must find a way to determine whether there are physiological or metabolic
2131 differences among cells, and more importantly, whether these relate to the
2132 experimental timepoint they are sampled from. It is however important to bear in
2133 mind that phenotypic heterogeneity is often highly unstable meaning that when
2134 cells are no longer exposed to the stressful conditions that select for the
2135 emergence of tolerant phenotypes, they often switch back. This has been
2136 demonstrated in populations of *S. aureus* whose small colony variants (SCV's),
2137 defined as 'slow growing phenotypic variants that forms pinpoint colonies when
2138 plated on agar, revert to normal size when sub cultivated (Vulin et al., 2018). This
2139 may limit our ability to identify and study differences among cells cultivated from
2140 experimental populations. If the ST131 cells that remain at the end of our
2141 experiment do indeed represent a subpopulation of cells in possession of distinct
2142 traits that permit their coexistence with Nissle, it may be possible to manipulate
2143 the fitness landscape to reduce the advantage of such phenotypes. In doing so,
2144 the eradication of the remaining population of ST131 can be achieved and
2145 recurrence of extraintestinal infections mitigated against.

2146 Patterns identified in the phage dynamics suggest that the presence of Nissle
2147 has a significant effect on the susceptibility of ST131 to phage infection (Figure
2148 3C). In our combination treatment, a peak in PFU/mL, approximately one order
2149 of magnitude greater than that reached in the absence of Nissle, is evident

2150 (Figure 3C). In this treatment, with the starting frequency of ST131 cells half of
2151 that present in the phage only treatment, there are fewer hosts available for
2152 phage to infect and as such we would predict phage to replicate to a lower final
2153 density at T1. However, in contrast to this prediction, phage in this treatment
2154 achieve a significantly higher PFU/mL providing some indication that the
2155 presence of Nissle increases the susceptibility of ST131 to phage infection
2156 (Figure 3C). Phage susceptibility, defined as the vulnerability of a bacterial target
2157 to active phage, can be described as a plastic trait (Perlemonie et al., 2021). The
2158 evolution of phage resistance is an example of such plasticity, with investment in
2159 resistance mechanisms such as CRISPR-Cas providing cells with a reduced level
2160 of susceptibility and subsequent protection from phage infection (Watson et al.,
2161 2019). However, this plasticity is not unidirectional, and the susceptibility of
2162 bacterial targets can also be increased. In our experiment, the increased
2163 susceptibility of ST131 may result from the action of a substance excreted by our
2164 probiotic competitor. This may act on the phage to increase its infective ability or
2165 on ST131 to increase its susceptibility to infection. The use of spent medium
2166 from our competition assays could be used to investigate this hypothesis. Filtering
2167 the medium to remove all traces of bacteria will leave any secreted products
2168 behind. By applying this filtrate to cultures of ST131 and phage, it will be possible
2169 to determine if the secreted products of Nissle are responsible for the altered
2170 susceptibility of ST131 to phage infection with the same peak in PFU/mL
2171 expected to be observed. Alternatively, the response of ST131 to the presence
2172 of a competitor may be responsible for the populations increased susceptibility to
2173 phage infection. For example, in bacterial populations, quorum sensing allows
2174 cells to communicate via chemical signals known as autoinducers to regulate the
2175 expression of genes such as those involved in competitive interactions (Pena et

2176 al., 2019). Cazares, García-Contreras and Pérez-Velázquez (2020) recently
2177 reported that there is a close association between quorum sensing and the
2178 susceptibility of bacteria to phage infection. In *E. coli* and *P. aeruginosa*, quorum
2179 sensing has been shown to promote phage susceptibility although the
2180 mechanisms remain largely unknown (Cazares, García-Contreras and Pérez-
2181 Velázquez, 2020). It is possible that ST131's use of quorum sensing to coordinate
2182 and modulate its response to the threat of ecological competition results in the
2183 unintended consequence of increased susceptibility to phage (Abisado et al.,
2184 2018).

2185 The spatial structure of the ST131 population promoted by competition may
2186 provide an alternative explanation. In response to ecological competition,
2187 bacteria are known to form clusters, aggregate together or form biofilms that offer
2188 some form of communal benefit or protection from competitors (Hernandez-
2189 Valdes et al., 2020; Abedon, 2012). Abedon (2012) coined the term 'spatial
2190 vulnerability' to describe the concept that certain cellular arrangements may act
2191 to increase the vulnerability of bacteria to phage predation. It is possible that the
2192 grouping of ST131 cells acts to increase the likelihood of encountering phage as
2193 the group acts as a larger target for host collision than any individual and spatially
2194 separated cell (Abedon, 2012). Once a cell within the group has been infected,
2195 the virions released from cell lysis are able to find and attach to a new host at a
2196 faster rate thus also leading to an increase in phage absorption rate (Abedon,
2197 2012). Targeted investigations that explore the spatial organisation of ST131
2198 promoted by the presence of interspecific competition would therefore be an
2199 interesting avenue for future research. It is also important that we recognise that
2200 the increased susceptibility of ST131 to phage when in competition with Nissle
2201 may be a result of the life cycle of phage H. Although we have had no indication

2202 that phage H is lysogenic, we cannot exclude the possibility that it might be. It is
2203 therefore possible that the peak seen in PFU/mL in our competition treatment
2204 may be a result of the induction of phage as a response to the external stressors
2205 imposed by competition with Nissle (Howard-Varona et al., 2017). It will be
2206 important to ascertain whether the increased susceptibility to phage that we have
2207 observed in this study is unique to the use of Nissle or whether it is replicated
2208 when using alternative probiotic strains and ST131-specific phage. Being able to
2209 explain the dynamics we see *in vitro* will be a crucial step to the implementation
2210 of our two-tiered strategy in clinical settings.

2211 Contrary to our hypotheses, the presence of a competitor did not reduce the
2212 rate at which phage resistance evolved among our target population (Figure 5).
2213 The influence of bacterial competition on the outcome of phage-host interactions
2214 must be considered when introducing phage into complex microbial communities,
2215 such as the gut microbiota (Cani et al., 2018). The presence of bacterial
2216 competitors can alter the strength of selection for resistance, typically via their
2217 effect on population demography (Mumford and Friman, 2017). Whether via
2218 indirect competition for resources or the direct production of bacteriocins,
2219 competitors are often responsible for a reduction in the population density of focal
2220 pathogens (Mumford and Friman, 2017; Levin and Bull, 2004; Lopez-Pascua and
2221 Buckling, 2008). The reduced phage encounter rates and lowered mutation
2222 supply rate that result, act to weaken the strength of selection for the evolution of
2223 resistance (Mumford and Friman, 2017; Levin and Bull, 2004; Lopez-Pascua and
2224 Buckling, 2008). We therefore hypothesised that a slower rate of resistance
2225 evolution would be observed in our φ + NIS treatment in comparison to φ. As
2226 evident in Figure 5A, a resistance assay conducted at timepoint three did not
2227 support this hypothesis, revealing that there was no significant difference in the

2228 level of phage resistance between the two treatments (Figure 5A). This suggests
2229 that contrary to our predictions, the presence of Nissle is strengthening the
2230 selection for phage resistance. This may be explained by our previously proposed
2231 hypothesis that the presence of Nissle increases the susceptibility of ST131 to
2232 phage infection. Regardless of the mechanisms responsible, the increased
2233 susceptibility of ST131 would be expected to strengthen the selection pressure
2234 for the evolution of resistance resulting in the similar rates of resistance evolution
2235 between the two treatments. Figure 3C demonstrates that as the resistance
2236 phenotype is established throughout the ST131 population, phage H is rapidly
2237 driven to extinction. With the extinction of phage, the φ + NIS and NIS treatment
2238 are now akin and we would therefore expect the frequency of ST131 to rise to
2239 the same level, however this is not what we observe (Figure 3A/4A) (Fazzino et
2240 al., 2020). The evolution of resistance could provide a reasonable explanation for
2241 this considering the widely documented fitness costs associated with it
2242 (Burmeister et al., 2020). However, the persistence of phage resistant ST131 at
2243 low frequencies within the population (Figure 3A/3A) as well as the absence of a
2244 competitive cost of phage resistance in the absence of phage (Figure 5B/5C) is
2245 indicative of a low, if not non-existent, cost of resistance to phage. Instead, it
2246 appears that the presence of phage in the early stages of competition exerts a
2247 lasting effect on the fitness of the ST131 population. Although the extinction of
2248 phage H from the population has removed the direct impact of phage predation
2249 on the fitness of ST131, secondary, indirect effects of the phage continue to
2250 interfere with the competitive interactions between ST131 and Nissle (Testa et
2251 al., 2019). These effects might be better understood if we consider the distinct
2252 limiting factors acting on the two populations. The density of Nissle is
2253 predominantly limited by the availability of resources allowing it to focus its

2254 energetic investment into improving its competitive ability. In contrast, the most
2255 direct threat faced by the ST131 population is cell lysis induced by phage
2256 infection. With a finite amount of resources, the ST131 population must prioritise
2257 its investment in the evolution of phage resistance allowing Nissle to gain a
2258 competitive advantage over ST131. The influence of positive frequency
2259 dependent selection known to act on the Nissle population (Barron et al., 2019)
2260 subsequently acts to constrain the ST131 population to a lower population density
2261 than would be achieved if it competed with Nissle in the absence of phage. One
2262 way to test this hypothesis, would be to repeat the competition assays with the
2263 addition of a treatment utilising a resistant ST131 colony isolated from glycerol
2264 stocks of the previous experiments creating a resistant φ + NIS treatment. If the
2265 competitive advantage presented to Nissle as a result of the conflicting selection
2266 pressures faced by ST131 is indeed responsible, we would expect that in this
2267 new treatment, the frequency of ST131 is able to reach frequencies comparable
2268 to that of the NIS treatment.

2269 Over the course of this study, we found no evidence of the cost of phage
2270 resistance, which may act to compromise the success of our decolonisation
2271 approach (Meaden, Paszkiewicz and Koskella, 2015; Gould and Lewontin, 1979;
2272 Lenski and Levin, 1985). Examples of trade-offs between phage resistance and
2273 competitiveness can be found throughout the literature such as the competitive
2274 disadvantage reportedly conferred by the acquisition of resistance to T2, T4 and
2275 T7 phage (Meaden, Paszkiewicz and Koskella, 2015; Lenski and Levin, 1985).
2276 However, several studies have also failed to find any evidence for the reduced
2277 fitness of resistant hosts (Meaden, Paszkiewicz and Koskella, 2015). The same
2278 variability among studies in the cost of resistance can be seen in those aiming to
2279 quantify the costs of resistance to antibiotics. Although there is substantial

2280 evidence that resistance is costly, there exist studies such as that by Dimitriu et
2281 al. (2019) which found no evidence of a fitness cost associated with the carriage
2282 of β-Lactamase encoding resistance gene $bla_{CTX-M-14}$ (Melnyk, Wong and Kassen,
2283 2015). To test for the cost of phage resistance, we compared the fitness of WT
2284 ST131 with the fitness of three different phage resistant colonies when in
2285 competition with Nissle, in the absence of phage (Figure 5B/5C). In doing so we
2286 assumed that the fitness cost of phage resistance would be expressed in terms
2287 of ST131s' reduced competitive ability but this assumption may not have been
2288 correct (Vogwill and MacLean, 2015). The trade-off associated with the
2289 development of phage resistance in ST131 may be paid instead, in the form of
2290 attenuated virulence or a heightened sensitivity to the immune system of its host,
2291 factors that our experimental design does not provide us with the capacity to test
2292 (Shen and Loessner, 2021; Friman et al., 2011). It is important to note that the
2293 fitness values of our WT ST131 and three phage resistant colonies were taken
2294 from two different experiments due to time constraints. It will be important that
2295 this experiment is repeated at the earliest opportunity and that fitness values for
2296 WT and resistant ST131 are obtained from the same experimental period and
2297 under the same experimental conditions before any conclusions are reached. If
2298 low or absent fitness costs continue to be observed, it will be crucial for us to
2299 identify whether this is replicated when using alternative ST131-specific phage or
2300 whether it is a direct function of the use of phage H and, if so, what features of
2301 phage H are responsible.

2302 Our data indicates that the evolution of phage resistance may confer a fitness
2303 benefit to our ST131 host. Comparison of WT, phage sensitive ST131 with three
2304 resistant colonies revealed a significant difference in relative fitness when
2305 competed against Nissle (Figure 5B/5C). The relative fitness of resistant colony

2306 1 and 2 was significantly greater than that of their WT counterpart. This is
2307 suggestive of the occurrence of a so-called trade-up where the evolution of
2308 resistance has a positive effect on elements of bacterial fitness (Burmeister et al.,
2309 2020). Investigations into the existence of trade ups have been largely focussed
2310 on the influence of phage resistance on antibiotic susceptibility with reported
2311 increases in resistance to antibiotics, such as tetracycline and albicidin, conferred
2312 by resistance to phage among *P. aeruginosa*, *E. coli* and *K. pneumoniae* strains
2313 (Moulton-Brown and Friman, 2018; Burmeister et al., 2020). Yet these examples
2314 contrast with studies that have reported the association between phage
2315 resistance and heightened antibiotic susceptibility, such as an increase in Colistin
2316 sensitivity in phage resistant *Acinetobacter baumannii* (Wang et al., 2021). This
2317 indicates that the influence of phage resistance on antibiotic susceptibility is
2318 bidirectional and forces us to ask the question of whether other components of
2319 bacterial fitness may also have the capacity, under certain conditions, to be
2320 traded-up (Scanlan, Buckling and Hall, 2015). To help understand whether the
2321 fitness advantage of phage resistant ST131 is the result of such a trade-up, it will
2322 be necessary to identify the mechanisms of resistance employed by ST131 in
2323 future studies. Interestingly, the effect of phage resistance on relative fitness was
2324 not consistent among all three of our resistant colonies, with the relative fitness
2325 of RES3 not significantly different from that of WT ST131. In contrast to RES1
2326 and RES2, this colony was isolated from our ST + NIS + φ treatment which may
2327 help to explain such variation. It is possible that in the presence of Nissle, ST131
2328 utilises an alternative mechanism of resistance that does not result in the trade-
2329 up we appear to observe for resistant colonies 1 and 2. Alternatively the absence
2330 of this trade-up may result from a trade-off that ST131 has had to make when
2331 evolving phage resistance at the same time as competing with an antagonistically

2332 strong ecological competitor. It may also be possible that the strength of
2333 competition between our three resistant colonies and Nissle, may have been
2334 asymmetric. Our ST131 colony (RES3) isolated from the ST + NIS + φ
2335 competition treatment differs from RES1 and 2 by the fact that it has already been
2336 exposed to competition with Nissle. It's possible that on secondary exposure, the
2337 strength of competition is increased which would likely cancel out any benefit or
2338 trade-up in relative fitness that ST131 may have experienced resulting from
2339 phage resistance. The idea that the interaction between Nissle and ST131 may
2340 be different in this treatment is aided by morphological data which shows that
2341 Nissle colonies present in competition with RES 3 appear to be morphologically
2342 distinct from those competing with RES1 and RES2 (Figure 2). Comparisons
2343 between Nissle colonies isolated from each competition may provide some
2344 explanation for the variation observed and help to predict how the influence of
2345 ecological competition may influence the outcome of interactions between phage
2346 resistance and bacterial fitness. In doing so we aim to prevent the probiotic
2347 application of phage from resulting in an unintended, and potentially harmful,
2348 increase in the pathogenicity of ST131 and enhance the likelihood of their uptake
2349 as therapeutic agents.

2350 Resistance to phage H did not provide cross-resistance to other ST131-specific
2351 phage suggesting the absorption receptors or resistance mechanisms used are
2352 distinct (Gu et al., 2012). Table 1 demonstrates the average PFU/mL of 10
2353 phages on bacterial lawns of ST131 isolates identified as resistant to phage H,
2354 susceptible to phage H and on WT EC958 and Nissle. Although within the scope
2355 of this study we were not able to establish the mechanisms responsible, the
2356 specificity of resistance to phage H is indicative of surface modification, the
2357 simplest and most common line of defence employed by bacteria against phage

2358 infection (Broniewski et al., 2020). It is widely acknowledged that when applying
2359 phage cocktails to prevent the development of resistance, cocktails composed of
2360 phage utilising different host receptors are preferred as it increases the likelihood
2361 that bacteria resistant to one phage will remain susceptible to others (Korf et al.,
2362 2020; Gu et al., 2012). The results presented in Table 1 are therefore
2363 encouraging and suggest it may be possible to develop a cocktail of a minimum
2364 of at least two phages from the ten we have selected. To do so, a Step-by-Step
2365 (SBS) approach will be implemented whereby co-culture of each phage with WT
2366 EC958 isolates will select for resistant mutants. Each phage will then be grown
2367 on lawns of these mutants to assess whether resistance is generalised or
2368 independent to the specific phage used for the selection of phage-resistant
2369 mutants. The use of this approach ensures that the resulting cocktails are
2370 comprised of phages that are lytic against our WT EC958 as well as resistant
2371 mutants and aims to enhance the therapeutic potential of phage cocktails for use
2372 against MDR bacterial infections (Gu et al., 2012). However, the use of phage
2373 that utilise different cell surface receptors does not necessarily provide protection
2374 against the evolution of cross-resistance with global regulator mutations able to
2375 confer generalist resistance through the simultaneous modification of multiple
2376 receptors (Wright et al., 2018). Additionally, lack of cross-resistance *in vitro* does
2377 not mean it won't evolve *in vivo*. In our experiment phage resistance was selected
2378 for by exposing susceptible isolates to each phage independently, conditions that
2379 are not representative of the application of a phage cocktail *in vivo*. It is therefore
2380 possible that the resistance mechanisms that have evolved *in vitro* will be
2381 different to those that evolve *in vivo* with the simultaneous exposure to multiple
2382 phages potentially selecting for the use of more generalised forms of resistance
2383 that our phage cocktail will be less effective against. The selection of phage

2384 combinations that do not select for cross-resistance must be incorporated into the
2385 cocktail design process and although caution must be taken in extrapolation their
2386 results, *in vitro* studies such as ours will prove useful in supporting this (Wright
2387 et al., 2018).

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# General Discussion

In Chapter one, we provide direction on how environmental sampling regimes may be designed to optimise the success of isolating broad host range phages. However, such direction is only beneficial if the value of host range determined at the point of isolation is stable. Understanding the degree of plasticity in the characteristic of host range is therefore necessary and will help determine the usefulness of phage as therapeutic agents. It is widely acknowledged that one of the main advantages of the use of phages over conventional antibiotics is their high host specificity, yet the stability of this trait is not well known. If phages are to be applied in areas of the body that harbour diverse communities of bacteria that are important for human health, such as the components of the gut microbiota, it is important to consider the likelihood of phage expanding their host range *in vivo*. Mapes et al. (2016) suggest that the host range of a phage is only ever applicable to the environment in which it was isolated from. Therefore, if you transfer a phage into a novel environment such as the gastrointestinal tract of a patient, the phage is likely to adapt its host range, it being a property that can evolve over time. We must therefore consider how much the environments in which phage are intended to be used may influence the likelihood of such viral host jumps occurring. Work by De Sordi, Khanna and Debarbieux (2017) reveals that the gut microbiota acts as a source of evolutionary pressure promoting the expansion of phage host range. They assessed the ability of phage P10 to infect previously insensitive host strains of *E. coli* and demonstrated that the diversity of hosts present in the microbiota promotes viral host jumps (De Sordi, Khanna and Debarbieux, 2017). If the environments in which phage are applied promote undesired host range expansion, it may be of benefit to investigate ways in which we can stabilise the property via selection for certain traits during phage isolation.

2415 One way in which we may be able to reduce the chance of undesirable host range
2416 expansion occurring *in vivo* is via the selection of phage that possess reduced
2417 evolutionary potential or evolvability. Bono, Draghi and Turner (2020) have
2418 suggested that generalist phage may be 'disadvantaged in evolvability' meaning
2419 that regardless of the host jumps available in the environment, they cannot
2420 access these pathways because of reduced genetic diversity. The ability of
2421 specialist and generalist phage to evolve to a novel host was compared and found
2422 that the number of mutations available to each phage that would extend their host
2423 range to include this host was considerably less for generalist phage, with only
2424 one or two mutations allowing such a host jump, versus the nine or more available
2425 to their specialist counterparts (Bono, Draghi and Turner, 2020). This suggests
2426 that prior niche expansion constrains future host range expansion and lowers the
2427 risk of the unintended infection of non-target strains as a result of reduced genetic
2428 diversity (Bono, Draghi and Turner, 2020). The identification of environments and
2429 isolation hosts that result in the preferential isolation of broad host range phage
2430 may not only improve the effectiveness of therapy but may also act to stabilise
2431 the property of host range.

2432 Although the focus of Chapter one is largely on identifying conditions conducive
2433 to the capture of naturally occurring broad host range phage, opportunities to
2434 artificially manipulate host range may provide an attractive alternative. With
2435 continuous advancements being made in bacteriophage-based biotechnologies,
2436 there is increasing interest in the potential for the use of genetic engineering to
2437 artificially manufacture broad host range phage (Santos and Azeredo, 2019).
2438 Genetic engineering would provide the opportunity to manipulate the host range
2439 of phage, via modifications to host-range-determining-regions (HRDR's) such as
2440 receptor binding proteins typically located on the tip of the phages tail (Yehl et al.,

2019; Dunne et al., 2021; Tremblay et al., 2006). Inspired by the application of genetic engineering to alter the specificity of therapeutic antibodies, Yehl et al. (2019) set out to engineer the host range of the phage T3 and was successful in extending its host range to infect phage-resistant bacterial mutants (Yehl et al., 2019). Alongside the expansion of phage host range, Yehl et al. (2019) were also able to engineer phages to possess traits that enable the suppression of the evolution of bacterial resistance, for instance. Such traits would be of extremely high value in phages intended to be used in clinical settings and highlights the vast potential of genetic engineering beyond its ability to manipulate host range. Previously we discussed that the host range of a phage can influence its evolvability, with generalists shown to possess lower levels of genetic diversity. For the purposes of genetic engineering, it would be beneficial to isolate those phages that possess a greater capacity for host range expansion which is offered by the greater genetic diversity, and subsequent plasticity, of narrow host range phage. This further emphasises the importance of the consideration of the intended application of phage on the isolation preferences imposed. Although the preferences of phage Isolation for the purposes of genetic engineering diverge from those discussed in this paper, it does not detract value away from the findings discussed. The act of isolating phage from the environment and the subsequent sequencing and characterisation that has been conducted provides important information on the drivers of host range that can be used to inform engineering attempts. Whilst our scientific knowledge and understanding of phage biology continues to be challenged and built upon, environmental isolation and characterisation studies such as this remain vital, but undoubtedly the future of phage therapy will rely on novel methods of manipulation conducted in the lab

2466 to produce 'libraries of engineered phage with expanded host range' in the most
2467 rapid and effective way possible (Yehl et al., 2019).

2468 To ensure that our selection for broad host range is not traded-off with other
2469 desired features that are necessary for the effective translation of phage into
2470 therapeutic agents, we must explore the fitness costs associated with host range
2471 expansion. Antagonistic pleiotropy is thought to provide an explanation for the
2472 limitations on generalism, where unavoidable trade-offs mean that the expansion
2473 of host range will inevitably lead to a decrease in the infectivity of phages on their
2474 original hosts (Bono, Draghi and Turner, 2020). A study by Duffy, Turner and
2475 Burch (2006) demonstrates that although the influence of antagonism is evident
2476 in the majority of cases, there were also some mutations that allowed the host
2477 range of the RNA bacteriophage φ6 to expand without imposing any significant
2478 fitness costs (Duffy, Turner and Burch, 2006). In such circumstances it is possible
2479 that compensatory mutations may be acting to ameliorate any fitness costs that
2480 have been imposed as the existence of no-cost generalists is highly unlikely.
2481 Perhaps trade-offs at the microevolutionary scale are not as inevitable or visible
2482 as those seen at the macroevolutionary scale or perhaps these fitness costs are
2483 experienced but not within the short evolutionary time frame that experimental
2484 evolution studies are commonly performed. For example, Bono, Draghi and
2485 Turner (2020) suggests that the fitness costs may not be observed through the
2486 reduced infectivity of phage across the breadth of hosts they infect but rather
2487 through their reduced ability to adapt, otherwise referred to as reduced
2488 evolvability. Much like the paradox of the Darwinian demon, the apparent lack of
2489 existence of a super-phage able to infect every member of a species, supports
2490 the fact that fitness costs do in fact occur. If we are to focus isolation attempts on
2491 broad host range phages, we must set to find out how and when such fitness

2492 costs are experienced. An understanding of the costs of generalism and niche
2493 expansion also has broader implications for public health, for instance emerging
2494 and re-emerging pathogens are commonly host generalists (Zhao and Duffy
2495 2019). Understanding the fitness costs associated with niche expansion may
2496 allow us to ameliorate these in an attempt to encourage the evolution of broader
2497 host range phage and ultimately allow us to bring phages and other pathogens
2498 under greater human control.

2499 In Chapter two, we propose that resistance to phage H is likely to have evolved
2500 via surface receptor modifications which act to inhibit phage infection. Although
2501 surface modifications are thought to be the most widespread mechanism of
2502 acquiring resistance, many systems of bacterial defence are initiated post-
2503 infection (Isaev, Musharova and Severinov, 2021). The importance of these
2504 systems and the extent to which they may interfere with the application of phage
2505 therapy must form the basis of future investigations. Throughout this study, the
2506 susceptibly of ST131 to phage infection was determined using spot assays, with
2507 the presence of plaques considered evidence of successful phage infection.
2508 However, the infection of a host does not mean a phage is guaranteed to survive,
2509 replicate and successfully eliminate its host. The Prokaryotic Antiviral Defence
2510 LOCator (PADLOC) developed by The Jackson Lab is a software tool for
2511 identifying antiviral defence systems in prokaryotic genomes (Payne et al., 2021).
2512 The output for our ST131 reference isolate, EC958, revealed the presence of a
2513 variety of post-infection phage defence systems including abortive infection
2514 systems (AbiE) that inhibit the propagation of phage and result in the seemingly
2515 altruistic death of infected bacterium, retron Ec67 which has been shown to
2516 mediate defence against dsDNA phages, Bacteriophage Exclusion (BREX) which
2517 inhibits DNA replication of adsorbed phage, and Gabija, a newly described

2518 bacterial defence system (Dy et al., 2014; Millman et al., 2020; Gao et al., 2020;
2519 Goldfarb et al., 2015; Cheng et al., 2021). As methods, such as the administration
2520 of phage cocktails, help to improve the success of initial host infection, the more
2521 important these post infection defences will become. We will need to ensure that
2522 we understand the fitness costs associated with them, whether certain systems
2523 are more likely to be favoured in particular environments, such as the community
2524 context of the gut where ST131 is found, and whether the specialised nature of
2525 some of these systems, such as CRISPR-Cas, is likely to form a barrier to the
2526 widespread uptake of phage therapy. It is encouraging that counter evolutionary
2527 mechanisms have already been detected in phage including anti BREX and Anti-
2528 CRISPR mechanisms, but the success of phage therapy is almost certainly going
2529 to rely on the ability of phage to stay one step ahead in the bacteria-phage
2530 coevolutionary arms race (Isaev et al., 2020; Pawluk, Davidson and Maxwell,
2531 2018). The use of phage training which encourages the evolution of such
2532 mechanisms *in vitro* may be a useful approach in achieving this (Borges, 2021).

2533 Alongside bacterial resistance, human factors such as the public acceptability
2534 of phage therapy, represent a major barrier to its widespread implementation in
2535 the West (Oliveira et al., 2015; Jones, Letarov and Clokie, 2020). The uptake of
2536 phage therapy has been hindered by the preferential use of broad-spectrum of
2537 antibiotics as well as concerns over its clinical safety. Although interest in the
2538 therapeutic use of phage has increased substantially in recent years, there may
2539 still be a way to go before phage therapy forms a standard part of global modern
2540 medicine. The recent Covid-19 pandemic may prove another barrier in achieving
2541 this with the negative connotations that, for many, now surround the term virus,
2542 potentially creating a new challenge in patient consent. To tackle the AMR crisis,
2543 we must focus on both the biological and human factors that hinder the success

2544 of phage therapy and show an appreciation that the human factors at play are
2545 likely to differ based on the country and subsequent culture of the target
2546 population (Jones, Letarov and Clokie, 2020). The specificity of phage therapy
2547 also increases the risk of treatment failure and fuels further public hesitancy if the
2548 bacteria responsible for infection is misidentified or the incorrect cocktail
2549 administered. The controversy surrounding the safety of the MMR vaccine in the
2550 late 90's demonstrates just how destructive such hesitancy can be for the uptake
2551 of novel therapeutic agents and emphasises the importance of mitigating against
2552 such failures (Rao and Andrade, 2011; Jones, Letarov and Clokie, 2020;
2553 Anomaly, 2020).

2554 The application of an ecological, niche-based perspective to the administration
2555 of probiotics may help to improve their efficacy. Although the popularity of the
2556 consumption of probiotics has increased dramatically over recent years, their
2557 clinical benefit remains questionable with the majority of studies failing to provide
2558 evidence of the benefits of their use (Lerner, Shoenfeld and Matthias, 2019;
2559 Butler, et al., 2021). One of the main issues identified is their limited persistence
2560 in the gastrointestinal tract which can be explained if we consider the gut as an
2561 ecological community made up of a limited number of niches (Ojima et al., 2022).
2562 The availability of these niches for colonisation is determined by the composition
2563 of the hosts gut microbiota which is in turn influenced by a variety of factors
2564 including diet, environment, and age. The availability of niches is therefore likely
2565 to vary from person to person which can help explain why we often see
2566 differences in the response of individuals to probiotic administration. Drawing on
2567 existing and emerging ecological theory will be an invaluable way to improve our
2568 ability to predict the colonisation success of probiotic strains (Letten, Hall and
2569 Levine, 2021; Ojima et al., 2022). Low predicted success can then be buffered

2570 against through the application of strategies such as that suggested in this study
2571 where probiotics are used in combination with phage which acts to free up
2572 available niche space.

2573 The increasing prevalence of MDR, gram-negative bacteria is one of the most
2574 significant public health problems of the 21st century. With all of the WHO's critical
2575 priority pathogens belonging to this group, it is vital that the development of
2576 antimicrobials effective against gram-negative bacteria is the focus of current and
2577 future research (Barreto-Santamaría et al., 2021). Our study has contributed
2578 towards this through the exploration of alternative methods that may be used to
2579 target and eradicate *E. coli* ST131 (Forde, et al., 2019). We have demonstrated
2580 that phage capable of targeting this clone can be found across a range of sewage
2581 environments and have provided direction as to how sampling attempts can be
2582 optimised to achieve the isolation of clinically desirable, broad host range phage.
2583 To achieve this, we adapted the Shannon diversity index to provide a novel
2584 method for quantifying phage host range. We provide evidence that the use of
2585 this method enables us to identify subtle differences between phage in their
2586 infective ability that may be crucial in determining their therapeutic potential and
2587 demonstrate that such differences would go undetected using traditional
2588 measures of host range. To achieve the isolation of broad host range phage we
2589 recommend sampling from community wastewater environments with the use of
2590 multiple isolation hosts belonging to distinct phylogenetic backgrounds. To further
2591 understand the isolation preferences identified in this study, we encourage
2592 investigation into the characteristics of wastewater environments that promote
2593 the evolution of broad host range phage as well as into the phylogenetic
2594 relationships that have been inferred between the nine host isolates used in this
2595 study. Although our results provide clear support for phage as promising

2596 antibacterial and therapeutic tools, no single strategy can overcome the risks
2597 posed by antibiotic resistance (Abd-Allah et al., 2021). We therefore set out to
2598 explore the potential of the combined use of phage and probiotic strain *E. coli*
2599 Nissle for the control of ST131.

2600 In this study we have successfully demonstrated the value of combining
2601 selective bacteriophage and inter-specific competition in suppressing densities of
2602 *E. coli* ST131. In doing so we aim to provide support for the use of this form of
2603 combination therapy to achieve the intestinal decolonisation of ST131 in vivo. The
2604 benefits of such decolonisation would include a reduced risk of individual patients
2605 developing hard-to-treat, drug resistant infections, as well as the reduced
2606 prevalence and transmission of these microbes among the wider population. Our
2607 results provide great promise, with the combined use of phage and probiotic
2608 resulting in a significantly greater reduction in the density of ST131 than that
2609 achieved by either method alone. However, our research also raises several
2610 questions that should form the basis of continued research. Contrary to our
2611 theoretical expectations, our combination treatment failed to achieve the
2612 elimination of ST131. Considering that intestinal reservoirs are regarded as likely
2613 origins of extraintestinal infections such as UTIs and sepsis, the complete
2614 removal of ST131 is necessary to achieve effective clinical outcomes. It will
2615 therefore be necessary to conduct further experiments to help explain the
2616 persistence of ST131 observed in this study. Secondly, the efficacy of our
2617 combined antimicrobial strategy is centred on the fact that the evolution of phage
2618 resistance comes at a cost to bacterial fitness, however our results fail to support
2619 this, instead suggesting that resistance may in fact be beneficial. Further
2620 investigations are required to determine whether fitness costs differ when
2621 experiments are conducted in conditions more representative of those of the gut,

as well as into ways in which it may be possible to manipulate these costs to promote the elimination of ST131. Determining how the exposure of ST131 to a phage cocktail will alter the outcome of competitions and costs of resistance will also be a crucial step in the progression of this two-tiered strategy into *in vivo* settings.

Overall, the results of this study will provide valuable direction for the control and elimination of MDR pathogens. We have demonstrated that probiotics have the potential to play a vital role in the fight against antimicrobial resistance when used in conjunction with other antimicrobials and, in doing so, have highlighted the importance of combining ecological and evolutionary theory in the development of antimicrobial strategies. The use of such strategies to target and eliminate MDR pathogens, including but not limited to ST131, will result in a substantial reduction in the prevalence and recurrence of some of the most common and life-threatening infections, as well as the social and economic burden associated with them. Our research provides support to the growing body of evidence that suggests bacteriophages could present one of the most valuable weapons in our fight against AMR. Although further research is necessary to unlock their full therapeutic potential, bacteriophages provide us with a valuable opportunity to diversify our antimicrobial arsenal. Such diversity will leave us better equipped to deal with the threats imposed by AMR and provide hope that a return to the pre-antibiotic era can be averted.

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## Acknowledgments

I would like to offer my thanks to Dr. Ben Raymond and Dr. Stineke Van Houte for their continued support, guidance and feedback throughout this project. I would also like to thank Dr. Matthew Chisnall and Dr. Bridget Watson for their generous and patient assistance in the lab and their contribution to the development of the PEG precipitation protocol used in this study.

I would like to thank those at the Centre for Genomic Research at the University of Liverpool for conducting the sequencing and preliminary analysis of phage genomes supplied, as well as Anne Leonard and Matthew Upton for providing the *E. coli* strains used in this study. I would also like to recognise my colleagues at the University of Exeter for their support in the collection and provision of sample material.

I would like thank representatives from Healeys cyder farm, Treliske hospital and Falmouth sewage treatment plant, whose assistance in the process of sample collection was vital to this study.

Finally, I would like to thank my family and friends for their continued support and encouragement that has kept me going through it all.