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Research paper

Impact of bile salts on coevolutionary dynamics between the gut bacterium *Escherichia coli* and its lytic phage PP01

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ABSTRACT

Although host and parasites are typically embedded in complex abiotic and biotic environments our understanding of how environmental variation impacts on host-parasite interactions, including antagonistic coevolution (AC) is poorly understood. Nonetheless, previous studies using bacteria and bacteriophages have shown that variation in just one abiotic parameter can have profound effects not only on the type of AC dynamics observed but also the time-frames over which AC interactions can persist. Here, we investigated the effect of an important component of the abiotic human gut environment, bile salts, on AC dynamics between the bacterium *Escherichia coli* and the lytic phage PP01 in an *in vitro* model system. In the absence of bile salts *E.coli* and PP01 coevolved in a manner that is consistent with a directional arms race dynamic (ARD), with bacteria and phages evolving increasing resistance and infectivity ranges through time. However, in the presence of bile salts, evidence of directional coevolution was weaker and more variable across replicate communities. These effects may be explained, in part, by the negative effect of bile salts on both host and parasite population sizes; lower population sizes for both bacteria and phages will reduce encounter rates which in turn could mitigate the benefits of generalism in both host and parasite resistance and infectivity ranges that are observed for ARDs. The negative effect of bile salts on phage population size may also be partially independent of host population size as bile salts was found to negatively impact phage viability in the absence of bacteria, as well as reducing phage adsorption efficiency. Differences in bacterial morphological diversity between treatments were also noted, with the emergence of mucoid colonies in both bile salts and non-bile salts treatments but only in the presence of phages. These data contribute to the growing body of knowledge on how environmental variation can impact on interactions between hosts and parasites. More specifically, these results are particularly relevant to our understanding of how bacteria-phage interactions may be affected by different abiotic factors relevant to the complex environment of the human gut and have clear implications for the development of phage as therapeutics to target members of the gut microbiota and/or intestinal pathogens.

1. Introduction

Antagonistic coevolution (AC) between host and parasites is defined as the reciprocal evolution of host resistance and parasite infectivity and plays a key role in a range of ecologically and epidemiologically relevant processes including driving and maintaining genetic diversity within and between populations and the evolution of parasite infectivity and host resistance ranges (Brockhurst and Koskella, 2013; Hall et al., 2011; Woolhouse et al., 2002). To date, host-parasite AC has been largely studied *in vitro* using a number of different experimental models (Ebert, 2008; Koskella and Lively, 2009; Slowinski et al., 2016) with different species of bacteria and their lytic viral parasites, bacteriophages, collectively serving as one of the best studied host and parasite model combinations available (Betts et al., 2014; Brockhurst et al., 2007; Middelboe et al., 2009; Mizoguchi et al., 2003; Perry et al., 2015). Crucially, their short generation times, genetic tractability and capacity for rapid evolution facilitates experimentation and testing of theory relating to AC over short time-scales with relative ease (Brockhurst et al., 2007; Buckling et al., 2009). However, in addition to their utility as model organisms, researchers are increasingly using bacteria and phages to generate data for its intrinsic value given their global abundance and diversity, together with their ecological, evolutionary and commercial relevance (Gomez et al., 2015b; Gomez and Buckling, 2011; Mahony et al., 2012; Middelboe et al., 2009).

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In natural ecosystems bacteria and phages are typically embedded in complex abiotic and biotic environments with both predicted to play important roles in determining the outcomes of host-parasite interactions including the potential for hosts and their parasites to undergo reciprocal evolution for resistance and infectivity (Cairns et al., 2017; Gorter et al., 2016; Koskella and Brockhurst, 2014; Laine, 2009; Thompson, 2009). With respect to the abiotic environment, previous *in vitro* studies using bacteria and phages have shown that variation in just one abiotic parameter can have profound effects not only on the type of coevolutionary dynamics observed but also the time-frames over which coevolutionary interactions can persist (Cairns et al., 2017; Lopez Pascua et al., 2014). Therefore, understanding how variation in the chemical environment impacts on the mode and tempo of bacteria-phage interactions is important to understanding coevolutionary dynamics and its consequences in more natural and biologically realistic scenarios, such as in the human gut (De Sordi et al., 2017; Gomez et al., 2015b; Koskella and Meaden, 2013). Like many other naturally occurring ecosystems, the human gut is host to a complex, dynamic and diverse microbiota that includes bacteria and phages (De Sordi et al., 2019a; Ogilvie and Jones, 2015; Shkoporov and Hill, 2019). The extent to which bacteria and phages can coevolve in the human gut remains an open question (Scanlan, 2017) but inference from sequence data (Minot et al., 2013; Sharon et al., 2013) and recent work on bacteria-phage interactions in murine models are promising indicators (De Sordi et al., 2017; De Sordi et al., 2019b).

The abiotic environment of the human gut is chemically complex and variable in space and recent *in vitro* experiments suggest one particularly important gut associated compound - bile salts - may have an important role to play in determining the outcome of interactions between bacteria and phages (Gabig et al., 2002; Scanlan et al., 2017). Bile is produced in the liver and stored in the gallbladder where it can then be secreted into the small intestine to play an important role in dietary fat solubilisation. Bile has a number of components including bile salts which are sodium and potassium salts of conjugated bile acids (Begley et al., 2005). Although bile salt concentrations vary between individuals and between fed and fasted states, total bile salt concentrations reported in the fed intestine range between 0.5 and 37 mM, with reported values averaging at 10 mM (Holm et al., 2013). Interactions between bile and the bacterial component of the gut microbiome are well recognised and both the biotransformation and modification of bile by bacteria and bile mediated effects on gut bacteria play important roles in human physiology, metabolism and health (Begley et al., 2005; Enright et al., 2018; Long et al., 2017). Recent work has also shown that bile salts can have a diverse range of effects on and potential consequences for bacteria-phages interactions (Scanlan et al., 2017). This includes two opposing effects on bacterial population size. Bile salts can directly inhibit bacterial growth (thereby also reducing the supply of susceptible hosts for phage propagation). Conversely, bile salts can also inhibit phage mediated lysis of bacteria, thereby mitigating the negative effects of phages on bacterial population size and reducing the strength of natural selection for bacteria to evolve resistance to phages (Scanlan et al., 2017). Although these effects are both phage specific and dependent on bile salt concentration, they highlight the importance of the abiotic environment in determining the outcome of interactions between bacteria and phages. Nonetheless, it remains unclear how exposure to bile salts affects longer-term interactions between bacteria and phages, *i.e.* bacteria-phage coevolution. An understanding of bacterial coevolution with phages in the gut also has potentially important clinical and therapeutic implications. Resistance evolution to phages can select for phenotypic variation in bacterial hosts, including LPS variation and mucoidy (Betts et al., 2016; Scanlan and Buckling, 2012; Scanlan et al., 2015b). Phage selection for these clinically relevant phenotypes indicates that coevolution with phages has, in principle, the capacity to promote or attenuate within-host bacterial virulence (Leon and Bastias, 2015; Scanlan 2017; Scanlan et al., 2015a).

In order to investigate the impact of bile salts on coevolutionary interactions between bacteria and phages we ran an *in vitro* coevolution experiment using a strain of the gut bacterium *Escherichia coli* (NCTC12900) together with its infective lytic phage PP01 that have been shown to undergo multiple rounds of AC *in vitro* in nutrient rich broth (LB) in a continuous culture model (Mizoguchi et al., 2003). We adapted this model to a batch culture serial transfer model of coevolution with two coevolution treatments consisting of bacteria and phage inoculated into nutrient rich media with and without bile salts (0.5% w/v) and two corresponding control treatments (no phage, with and without bile salts). This model was run for 11 transfers (264 h, ~77 generations) to assess how bile salts, which are an ecologically relevant component of the gut abiotic environment, affected 1) the extent of bacteria-phage AC in terms of changing bacterial resistance and phage infectivity and 2) the qualitative dynamics of bacteria-phage AC (*e.g.* directional *vs* non-directional changes in resistance and infectivity; variation across replicate communities in each treatment). Our results show that in the absence of bile salts *E. coli* and phage PP01 coevolved in a manner consistent with a directional arms race dynamic (ARD), with bacteria and phages evolving increasing resistance and infectivity ranges through time. In the presence of bile salts, evidence of directional coevolution was weaker and more variable across replicate communities. Further quantitative analysis suggested this observation may be explained, at least in part, by the negative impact of bile salts on both bacteria and phage population sizes, which in turn may reduce their encounter rates such that the benefits of generalized resistance and infectivity phenotypes are reduced.

2. Material and methods

2.1. Strains and culture conditions

We used *Escherichia coli* NCTC12900 and the lytic phage PP01 for our coevolution experiment (Mizoguchi et al., 2003). *E. coli* NCTC12900 is a gram negative non-pathogenic strain, phenotypically similar to the toxigenic strain of *E. coli* O157:H7 but lacking verotoxin production (Woodward et al., 2003). This strain was kindly provided by Dr. Kay Burgess, (Ashtown, Teagasc, Ireland). PP01 is a lytic phage that was originally isolated from swine stool (Morita et al., 2002) and kindly provided by Prof. Yasunori Tanji (Tokyo Institute of Technology, Tokyo, Japan). PP01 is an unclassified Myoviridae, most closely related to T-even type coliphages and uses the host outer membrane protein C (ompC) and LPS as receptor sites for successful infection (Mizoguchi et al., 2003; Morita et al., 2002). *E. coli* and phage PP01 were propagated in LB broth (Sigma, Ireland) at 37 °C and phage were enumerated using soft agar overlay assays (0.6% agar w/v) prior to the coevolution experiment.

2.2. Coevolution experiment

Our coevolution experiment consisted of four treatments with 6 replicate communities per treatment; 1) *E. coli* propagated in LB, 2) *E. coli* and PP01 propagated in LB, 3) *E. coli* propagated in LB supplemented with bile salts (Sigma, Ireland) to a final concentration of 0.5% w/v and 4) *E. coli* and PP01 propagated in LB supplemented with bile salts to a final concentration of 0.5%. We chose a bile salt concentration of 0.5 w/v % (12 mM) as it is within the biological range of total bile salt concentrations reported in the fed intestine (~10 mM) (Holm et al., 2013). Experiments were initiated by inoculating bacteria (~10^8 colony-forming units) and phage (~10^5 plaque-forming units; for treatments 2 and 4 only) into 50 ml Falcon tubes containing 6 ml of nutrient broth as appropriate for the treatment as outlined above. Communities were incubated statically at 37 °C and at 24 h intervals (one transfer cycle (1 T)) 1% of each community was transferred to a new falcon tube containing fresh nutrient broth (supplemented with bile salts as appropriate). Optical density (OD) readings were recorded at each transfer using a 96-well microplate reader with a wavelength setting of 620 nm (MultiSkan FC, Thermo Scientific) and these data served as a proxy for bacterial population densities. We also estimated phage population densities at T1, T6 and T11 by spot plating serial diluted phage samples (phage isolation described below) on soft agar overlays containing the wild type *E. coli* NCTC12900 as a host, and we did this in triplicate. The experiment was run for a total of 11 transfers (264 h) and communities were stocked in 20% v/v glycerol at −80 °C at regular intervals.

To test for AC between the bacteria and phage in each community, we first isolated samples of phage at multiple times points. We did this for a given community at a given time point by treating a 1 ml aliquot from the experiment with chloroform (10% v/v), vortexing it prior to centrifugation and storage at 4 °C. Next, we isolated 20 bacterial colonies from each community at T1, T6 and T11. We did this by plating populations on LB agar plates and randomly picking 20 colonies, the latter only for phage treatments. Dilutions plated ranged from 10^{-2} and 10^{-8} to account for variation in population sizes between populations and treatments. This also allowed us to examine variation in *E. coli* colony morphology that has been shown to arise during coevolution with PP01 (Mizoguchi et al., 2003). Different colony morphologies were noted and counted to determine their population size within that specific population and treatment. We then tested the susceptibility of every bacterial colony isolate from transfer 6 (T6) to infection by sympatric phage samples (from the same replicate community) from the past (T1), present (T6) and future (T11). We also tested whether phage samples from transfer 6 (T6) in each community could infect sympatric bacterial colony isolates from the past (T1), present (T6) and future (T11). Host resistance was determined by streaking assays as described previously (Buckling and Rainey, 2002a; Scanlan and Buckling, 2012). Briefly, bacteria were streaked across a perpendicular line of phages that had been dried onto LB plates. We then scored bacteria as resistant (no visible inhibition by phages) or sensitive (visible inhibition) after overnight incubation at 37 °C, including the ancestral strain as a control on all plates. These data allowed us to investigate coevolutionary dynamics by measuring changes in bacterial resistance (proportion of 20 colony isolates that could not be infected by a given phage sample in a streaking assay) as a function of phage time (past, present, future), and phage infectivity (proportion of 20 colony isolates that could be infected by a given phage sample) as a function of bacteria time (past, present, future) (Buckling and Rainey, 2002a; Scanlan and Buckling, 2012). As described elsewhere (Buckling and Rainey, 2002a), under directional selection for increasing resistance and infectivity, we expect phage from the future to be relatively infectious, and bacteria from the future to be relatively resistant.

2.3. Effect of bile salts on phage survival

The effect of bile salts on phage survival outside of the bacterial host was assayed by incubating a pure stock of phages in LB or LB supplemented with bile salts (0.5% w/v) (n = 5 in both groups), at 37 °C for 24 h. Phages were then enumerated by serial dilution and soft agar overlay plaque assays using *E. coli NCTC* 12900 wild-type as the host.

2.4. One-step growth curve

A one-step growth curve of phage PP01 on the ancestral bacterial host was conducted in the presence and absence of 0.5% bile salts according to standard methods (Kropinski, 2018). In brief, bacteria were inoculated into Falcon tubes containing LB or LB supplemented with 0.5% bile salts and grown to an OD of ~0.5 (10^8 cfu/ml). These cultures were then inoculated with phage PP01 at a multiplicity of infection (MOI) of 0.01. After 5 min, each culture was serially diluted by a factor of 10, 100 and 1000, and phage titre in the diluted cultures was then measured at five minute intervals over a 90 min time-frame. To measure adsorption rate or proportion of unbound phage, samples were treated with chloroform prior to enumeration.

2.5. Statistical analyses

We tested whether coevolutionary dynamics differed between the two treatments using time-shift analysis (Gaba and Ebert, 2009; Gomez et al., 2015a; Lopez Pascua et al., 2014). First, we tested for directional changes in phage infectivity by determining whether bacteria sampled at T6 in each treatment were more resistant to phage samples from the past, present or future (T1), (T6) or (T11). We did this using linear mixed effects models fitted separately for each treatment with maximum likelihood (*lme* function in the nlme package of R version 3.3.3), with resistance (the proportion of 20 phages that bacteria were resistant to; arcsine transformed) as the response variable, phage time relative to bacteria time (past, present or future; fitted as a continuous linear term) and the interaction between phage time and bacterial replicate population (the six individual populations in each treatment; this interaction term means we fit six separate slopes) as predictors, and bacterial replicate population (the six individual populations in each treatment) as a random effect. Next, we computed two additional estimates of the strength of the directional change in bacterial resistance across phage samples from the past, present and future in each population. First, we took the slope of resistance across phage time (past, present, future) in each community. Second, we took the ratio of the total change in resistance between past and future phage samples relative to the sum of individual differences (past-*vs*-present and present-*vs*-future, expressed as absolute values), following (Andreazzi et al., 2017). This metric equals 1 when resistance increases monotonically across phage time, −1 when it decreases monotonically, and is closer to zero when resistance changes non-directionally across phage time. We then used *t*-tests to determine whether these measures of directional change differed on average between communities evolved in the presence and absence of bile salts. We also carried out equivalent analyses for variation of phage infectivity, for phage samples from T6 tested against bacteria from the past, present and future (T1, T6 and T11).

To determine treatment effects on bacterial biomass during the coevolution experiment, we used a linear mixed effects model with OD as the response variable, bile salts (with/without), phage (with/without) and time (as a categorical predictor) as factors, and replicate community (1–6 in each treatment) as a random effect. We fitted the model using maximum likelihood and simplified it by likelihood ratio tests comparing full and reduced models, and tested the significance of factors in the minimal adequate model using *F*-tests. We excluded two populations from the bacteria-only treatment after 168 h because they became contaminated with phages. We used an equivalent model for phage abundance over time (in the phage treatments only), with phage abundance (log-transformed plaque-forming units per ml) as the response variable, bile salts (with/without) and time as factors and population as a random effect. To compare phage viability in cultures incubated in the absence of bacteria with and without bile, we used a *t*-test.

3. Results

3.1. Bile salts affects bacteria-phage coevolutionary dynamics

In communities that coevolved in the absence of bile salts, bacterial resistance was higher against phages from the past than those from the future (effect of phage time: $F_{1,6} = 27.26$, $P = .002$; Fig. 1A). This indicates directional selection for increasing phage infectivity over time. The same was not true on average in the bile salts treatment (effect of phage time: $F_{1,6} = 0.26$, $P = .63$; Fig. 1B), indicating directional selection on phage infectivity was weaker here. This interpretation is supported by comparing the two measures of directional change that we computed for each replicate community in both treatments: the slope of resistance across phage time was negative on average in the absence of bile salts but closer to zero in the presence of bile salts (mean ± s.d. = −0.043 ± 0.035 and 0.013 ± 0.037 respectively,

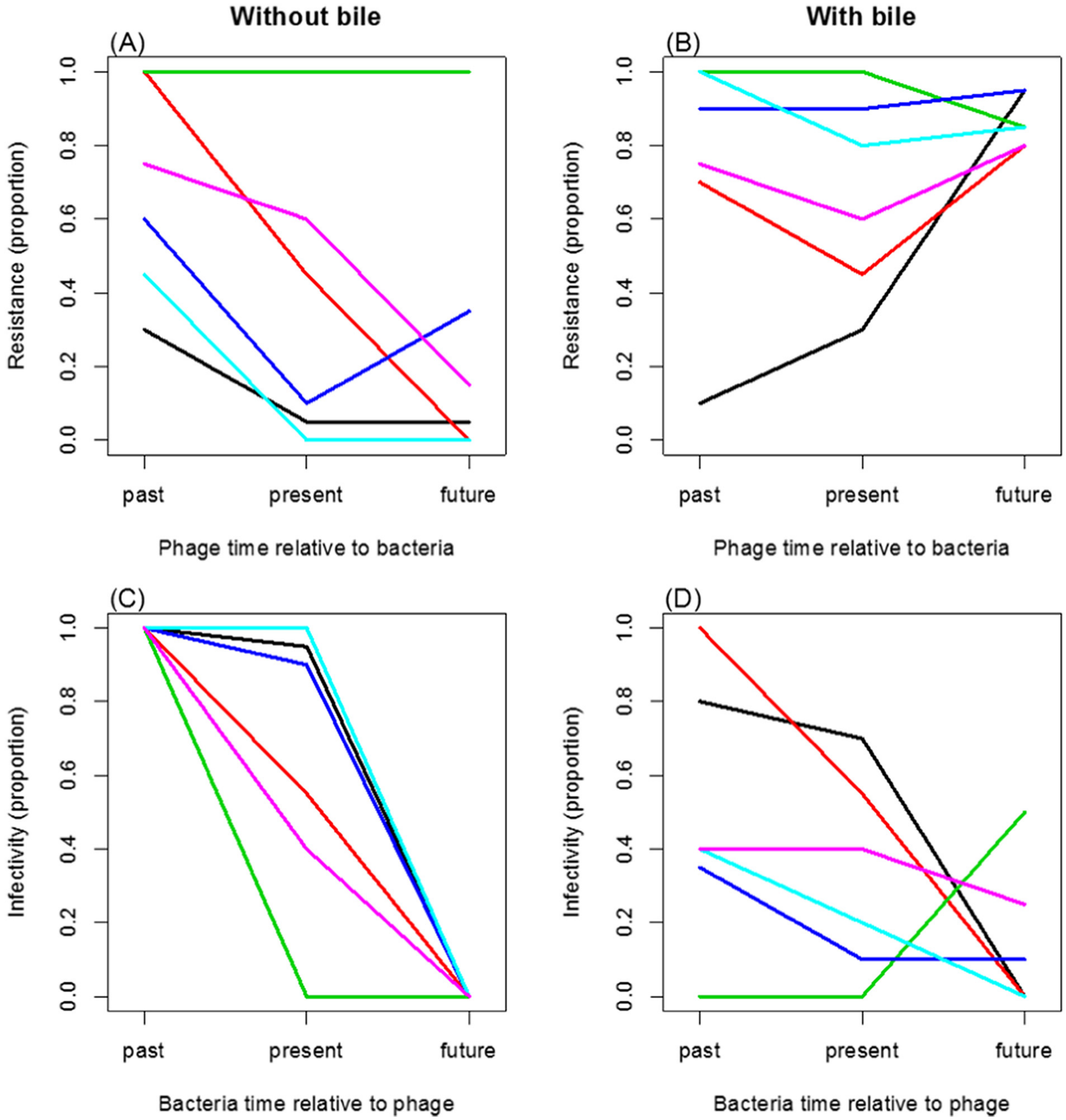

Fig. 1. Coevolutionary dynamics with and without bile salts. (A,B) Resistance of bacteria isolated after 144 h from each of six coevolving communities (shown by different coloured lines) tested against sympatric phages isolated from the same time point (present), an earlier time point (24 h: past) or a later time point (264 h: future). (C,D) Infectivity of phages isolated from 144 h from each of the same six communities tested against sympatric bacteria from the present, past and future.

Welch's $t_{9.96} = 2.65$, $P = .02$), and the ratio of total change compared to individual differences showed a similar pattern (mean ± s.d. = −0.87 ± 0.30 and 0.12 ± 0.82 in the absence/presence of bile salts, with one population excluded in the absence of bile salts due to no change over time; Welch's $t_{6.52} = 2.75$, $P = .03$).

On the bacterial side, those from the future were harder for phages to infect than those from the past in all six communities that coevolved in the absence of bile salts ($F_{1,6} = 58.36$, $P = .0003$; Fig. 1C). This indicates directional selection for increasing bacterial resistance over time. In the presence of bile salts, there was also an average decline in infectivity across bacterial samples from the past, present and future ($F_{1,6} = 30.92$, $P = .001$), but this was not consistent across replicate communities (time-by-population interaction: $F_{5,6} = 11.44$, $P = .005$). Again, this interpretation was supported by an effect of bile salts on the average slope of infectivity over bacteria time, a measure of directional change in bacterial resistance to infection (mean ± s.d. slope across bacteria time = −0.1 ± 0 and −0.035 ± 0.05 in the absence and presence of bile salts; Welch's $t_5 = 3.01$, $P = .03$). The alternative measure of directional change we computed, the ratio of the total change to the individual differences between time points, did not differ on average between the two treatments (Welch's $t_5 = 1$, $P = .36$), because all 12 populations showed monotonic changes over bacteria time, with only one population in the bile salts treatment increasing and all others declining (Fig. 1D).

In summary, we found strong evidence of directional selection on bacterial resistance and phage infectivity in the absence of bile salts (consistent with an arms race dynamic), but in the presence of bile salts the evidence was weaker and more variable across replicate communities.

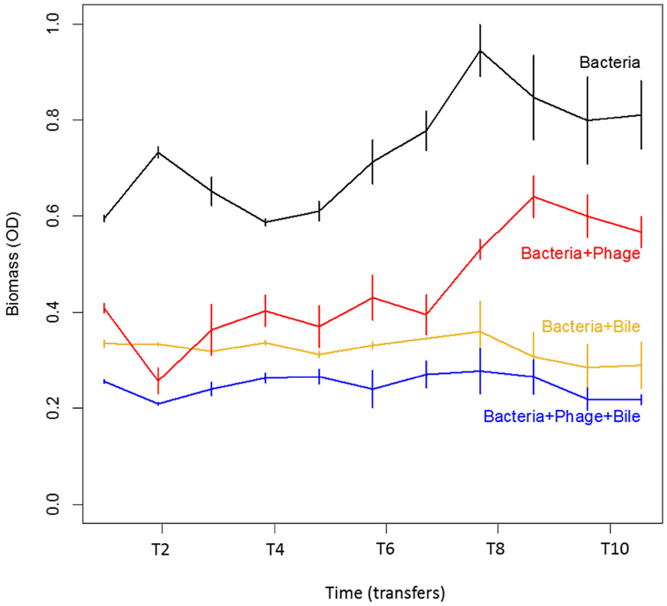

Fig. 2. Total biomass estimated by optical density (OD) over time in each of the four treatments (labelled at right). Each line gives the average of six communities ± s.e.

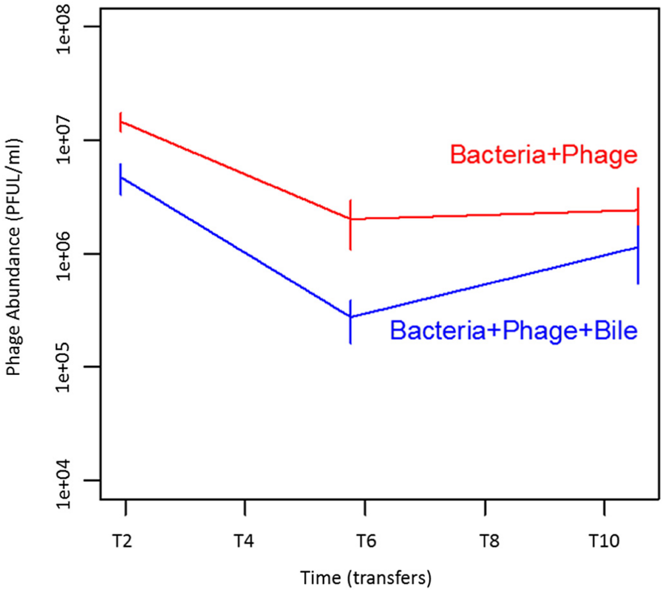

Fig. 3. Phage abundance over time, estimated by counting plaque-forming units (PFUs) at three time points. Each line gives the average ± s.e. for the six replicate populations in the presence and absence of bile salts (labelled at right).

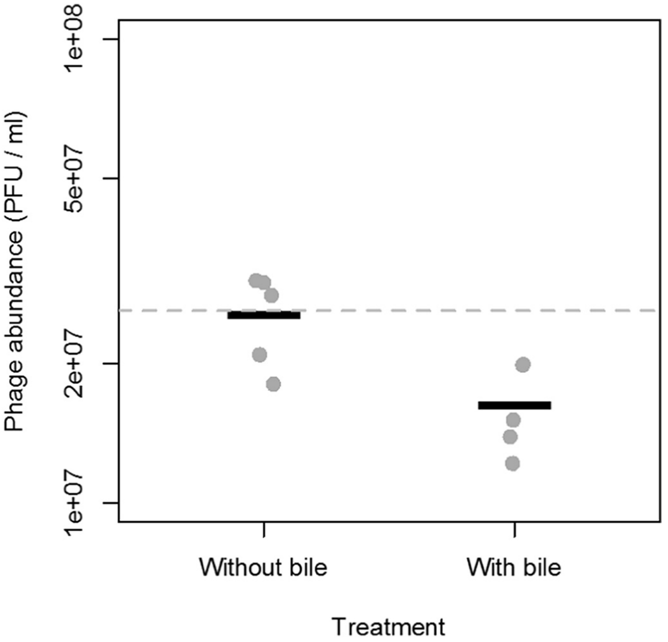

Fig. 4. Phage survival in the absence of bacteria with and without bile salts. Phage titre after 24 h incubation with or without bile salts is shown for five replicate populations (points) in each treatment. Bars show the mean for each treatment and the average titre at the start of the assay (from five technical replicates of the stock solution that was used to inoculate all assay replicates) is shown by the grey dashed line.

3.2. Variation of bacterial abundance across treatments

Adding bile salts reduced bacterial biomass measured by optical density during the experiment (effect of bile in a linear mixed-effects model: $F_{1,20} = 273.53$, $P < .0001$; Fig. 2). Phages also reduced biomass over time in both the presence and absence of bile salts (effect of phages in a linear mixed-effects model: $F_{1,20} = 89.26$, $P < .0001$; Fig. 2). Consequently, bacterial biomass in the communities that coevolved in the presence of bile salts was much lower than in those that coevolved in the absence of bile salts, and this was true across all time points.

3.3. Variation of phage abundance and survival across treatments

Adding bile salts reduced phage abundance in coevolving communities (effect of bile salts in a mixed effects model of phage abundance over time: $F_{1,10} = 5.63$, $P = .039$; Fig. 3). This could potentially be explained by the negative effects of bile salts on bacterial abundance (observed above in the absence of phage), which could reduce phage replication by limiting the supply of susceptible hosts. However, bile salts might also influence phage abundance directly by reducing survival outside the host. We found that after 24 h incubation in bacteria-free medium with or without bile salts, phage titre was reduced in the bile salts treatment relative to the non-bile salts treatment (Welch's $t_{6.90} = 3.10$, $P = .02$; Fig. 4), and had decreased from the start of the assay.

3.4. Bile salts reduce phage adsorption efficiency

A number of attempts at the one-step growth curve were made in an effort to compare the burst size of PP01 phages in the presence and absence of bile salts. We were unable to accurately infer burst size in these experiments because we found that phage adsorption was reduced in the presence of bile salts: ~100% of phages bound to the host within 5 min in LB media without bile salts, but in the presence of bile salts only ~50% of phages had adsorbed over the same time-frame and in the same experimental conditions (See Supplementary Fig. 1). This shows bile salts affected a key phage life history parameter (adsorption), but because it violated a key assumption of the one-step growth curve experiment (that 90–95% of phages adsorb to the host cells within 5 min (Kropinski, 2018)), we did not use these data to estimate burst sizes.

3.5. Morphological diversity

Variation in colony morphology was also recorded for colonies plated on LB for all four treatments at T1, T6 and T11. In total, three different colony variants consistent with morphologies described in a previous study (Mizoguchi et al., 2003) were noted here. The wild-type (WT) colony type (round, flat and smooth) dominated the vast majority of populations for each treatment at all time-points recorded, Supplementary Fig. 2 (A–C). However, the emergence of mucoid (M) colonies (irregular, glossy, raised colonies on LB (Mizoguchi et al., 2003)) was noted for a number of populations in both the bile salts and non-bile salts treatments but only in the presence of phages. Mucoids were first noted at T6 for one and four populations within the non-bile salts phage and bile salts phage treatments, respectively, albeit at a lower frequency than the WT colony morphology, see Supplementary Fig. 2B. For the non-bile salts phage treatment the mucoids were again noted in the same and one additional population at T11, see Supplementary Fig. 2C. In the bile salts phage treatment at T11 mucoids were detected in four of the six populations, however, its presence in specific populations for this treatment was, in part, inconsistent with data recorded from the previous time-point; mucoids were not detected in one population that was previously positive but was detected in a population that was previously negative. However, the absence of mucoids through time could be a sampling issue as, with one exception, mucoids were present at a lower frequency than the WT morphology and accordingly could be missed due to the thresholds of detection associated with serial dilution and plating. As outlined, colonies for the streaking assay were randomly selected and, depending on the time-point and population, mucoids were sometimes included. As observed for the WT phenotype, we noted variation in the individual resistance profiles for this phenotype when isolates were tested against phage from different time-points. The final morphology that was noted was consistent with the previously described Ss (small, round and smooth) morphology (Mizoguchi et al., 2003). However, this morphology was noted only at T11 for one population in the non-bile salts phage treatment, see Supplementary Fig. 2C.

4. Discussion

Although we have limited knowledge of how the abiotic environment impacts host parasite interactions and coevolutionary dynamics, previous work has shown that it not only affects the potential for and persistence of AC but it is likely to play an important role in determining the dynamics of AC (Cairns et al., 2017; Lopez Pascua et al., 2014). Our lack of knowledge on how interspecies interactions such as AC are shaped by variation in the environment is in part due to the complexity of natural ecosystems. However, the use of *in vitro* models circumvents this problem and provides the opportunity to explicitly test the potential effects of specific components of the abiotic environment on biotic interactions, such as bacteria-phage AC, under controlled settings (Brockhurst and Koskella, 2013; Brockhurst et al., 2007). Here, we found that a ubiquitous and variable component of *E. coli*'s natural environment, bile salts, strongly altered the coevolutionary dynamic between *E. coli* and a lytic phage, resulting in more variable and less directional coevolution compared to the same bacteria-phage combination in the absence of bile salts.

In line with earlier work (Mizoguchi et al., 2003) populations of both *E. coli* and phage PP01 persisted for the duration of the experiment (264 h) in LB nutrient rich broth and coevolved in a manner consistent with an Arms Race Dynamic (ARD). Bacteria were more resistant to phages from the past than to contemporary or future phages. Phages were more infective to bacteria from the past than to contemporary and future bacteria (Fig. 1A and C). Although bile salts did not impact on the persistence of *E. coli* and phage PP01 as both co-existed for the extent of the experiment, the average signal for ARD was weaker in the presence of bile salts (Fig. 1B and D), and the dynamics were more variable across replicate communities in the bile salts compared to non-bile salts treatments. Previous experimental and theoretical work has shown that a weakened arms race dynamics can be explained by reduced population sizes of host and parasites under lower nutrient conditions, which results in lower contact rates between hosts and parasites and thus negates the benefits of generalized resistance/infectivity phenotypes typical of ARDs (Lopez Pascua et al., 2014). Here we show that an analogous shift in dynamic can result from exposure to a key abiotic variable that *E. coli* and its phages will encounter in the GI tract. The significantly lower bacteria and phage population sizes in the bile salts compared to the non-bile salts treatment (Figs. 2 and 3) is likely to greatly lower their contact rates in a static environment. Therefore the negative impact of bile salts on both host and parasite population sizes may, at least in part, explain the dampened and more varied signal for ARD between the two treatments. Another non-mutually exclusive possibility that may explain the difference in ARD signal between the two treatments relates to mutation supply; given the necessity of a continuous mutation supply (as a function of population size and mutation rate) to support ongoing ARD the negative effect of bile salts on both bacteria and phage population size may also be relevant in this regard. However, this explanation is less likely given that we observed extensive changes in both resistance and infectivity trait evolution in the bile salts treatment and although bacteria and phage populations were significantly reduced in the bile salts compared to non-bile salts treatments the populations sizes of both were still quite high ($\sim 10^5$ phages per ml and $\sim 10^6$ bacteria per ml).

In addition to the effects of bile salts on AC interactions between bacteria and phages, bile salts also negatively affected phage viability and phage adsorption. PP01 phage exposed to bile salts had an almost 40% drop in viable infective phage particles after 24 h in the absence of a host (Fig. 4). This finding is consistent with previous work showing bile salts can negatively affect viability for some but not all phages tested (Scanlan et al., 2017) and has important implications for extra-host survival in the complex environment of the gut. Although phage numbers were lower in the bile salts compared to the non-bile salts treatment, there was clearly a sufficient supply of susceptible hosts for phages to continually propagate and thereby minimise the negative effects of exposure to bile salts in this *in vitro* model. However, in the more complex and spatially structured environment of the gut where a constant host supply may be limiting, this result might indicate that in such a scenario phages that are sensitive to bile salts are potentially at a greater risk of extinction than bile salt insensitive counterparts. Although we could not generate data on phage burst size when hosts were grown in the presence of bile, we did find that bile negatively affected phage adsorption to the host, with a ~50% drop in phage adsorption efficiency between bile and bile-free treatments. While the mechanistic basis for this effect is unknown, the most likely explanation is that bile salts alters some aspect of bacterial physiology such as the expression of phage binding targets and/or bile salts impact the phage itself (*e.g.* affects tail fibre structure or function). Elucidating whether this negative effect of bile salts on phage adsorption is a general phenomenon warrants further investigation, but this finding, together with the effects of bile salts on phage viability, have clear implications not just for understanding the ecology and evolution of phage-bacteria dynamics in the gut but also for phage therapy. For example, for phages that are being tested as therapies for gastrointestinal infections (Brussow, 2017) or as potential agents to modify the gut microbiome, it may be beneficial to also evaluate their capacity to survive in the absence of bacteria and in the presence of bile salts, as well as their adsorption efficiency at a range of biologically relevant concentrations.

Finally, coevolution with phages is known to drive variation in bacterial host morphology (Buckling and Rainey, 2002b; Scanlan and Buckling, 2012) and initial studies of AC between *E. coli* NCTC12900 and phage PP01 noted that a number of different morphological mutants emerged over the course of their 200 h experiment in continuous culture including the appearance of mucoid bacteria at 56 h post inoculation (Mizoguchi et al., 2003). Here, we also observed mucoids for both bile salts and non-bile salts treatments but this phenotype only emerged under phage predation (Supplementary Fig. 2). Interestingly, this phenotype has been observed for other species of bacteria *e.g. Pseudomonas fluorescens* and *Pseudomonas aeruginosa* in similar experimental models as a response to phage predation and has have been demonstrated to confer a generalist phage resistance phenotype (Betts et al., 2016) or provide a form of partial resistance thus facilitating apparent competition with phage sensitive counterparts (Scanlan and Buckling, 2012). In the gut environment exopolysaccharide producing phenotypes have been shown to play an important role in evasion of host immune responses as well as pathogen exclusion (Fanning et al., 2012). Importantly, our data indicates that bile salts does not constrain the evolution of a mucoid phenotype in response to phage predation and mucoids appeared to be more widespread in the bile salts-phage compared to non-bile salts-phage treatment by the end of the experiment (Supplementary Fig. 2). Nonetheless, whether mucoids evolve in response to phage predation in the more complex environment of the gut is unknown, but if they can, this supports the idea that antagonistic coevolution between bacteria and phages could have important indirect effects on host (human)-microbe interactions (Scanlan, 2017).

Declarations of interest

None.

Conflict of interests

All authors have no conflicts of interest.

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Appendix A. Supplementary data

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