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Phage-steering permits antibody-mediated clearance of *E. coli* K1 from the gut

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Abstract

E. coli strains expressing the capsule serotype K1 (*E. coli* K1) are a prevalent cause of neonatal sepsis and meningitis. The gut microbiota of healthy adults is a natural reservoir of *E. coli* K1, from which it can spread to extra-intestinal sites or be transmitted from mother to infant during birth. Accordingly, shifting gut colonization from potentially pathogenic E. coli strains to more benign strains could reduce the risk of disease. Here, we leverage selective pressures exerted by bacteriophage and mucosal antibodies to limit gut colonization by E. coli K1 and prevent its transmission. K1-specific bacteriophages (phages) rapidly drive a within-host evolution of capsule-less mutants with exposed surface O-antigens. These mutants become susceptible to vaccine-induced intestinal IgA targeting the bacterial O-antigen, allowing competitive exclusion by the probiotic strain *E. coli* Nissle. In a murine vertical transmission model, 77% of pups were protected from transmission of *E. coli* K1 when the mother was vaccinated and treated with phages, whereas *E. coli* K1 was detected in most pups by day 10 of life when the mother received vaccination or phage therapy alone. Although the high diversity of *E. coli* makes generalization challenging, combining vaccination with phage-steering represents a promising approach for further exploration in eliminating infectious reservoirs.

Introduction

The gut microbiota is a complex ecosystem of microbes that play an important role in human health and development[1–3]. While many bacterial species provide direct health benefits to the host by liberating nutrients or maintaining immunological homeostasis[1,4], some are opportunistic pathogens and can cause life-threatening disease in extraintestinal sites[5–7]. In otherwise healthy adults, extra-intestinal pathogenic *Escherichia coli* (*E. coli*) are generally relatively harmless gut commensals[5]. Nevertheless, many of these strains harbor specific virulence factors that increase their ability to cause systemic infections [8–11].

One of the most relevant of these virulence factors is the K1 polysaccharide capsule produced by approximately 25% of *E. coli* causing extra-intestinal infections in adults [12]. The K1 capsule is a membrane-anchored polymer of α-(2-8)-linked sialic acid, forming poly-sialic acid (PSA)[13,14]. PSA is also a post-translational modification of the host neural cell adhesion molecule (NCAM), making the K1 capsule a self-antigen and thus non-immunogenic[15–17]. The K1 capsular polysaccharides mask underlying surface antigens, including the LPS O-antigen and outer membrane porins[15,18–22]. In addition, the K1 capsule enables immune evasion by inhibiting phagocytosis and by blocking the activation cascade of the complement system, promoting survival in the bloodstream[21,23,24]. The self-antigen-nature of this capsule

type limits the possibility of using vaccination to prevent intestinal colonization or systemic infections caused by *E. coli* K1[25]. Neonates are at increased risk of developing sepsis or meningitis because of the vulnerable intestinal architecture of the neonatal gut, with low amounts of protective mucus and decreased intestinal mobility and acid secretion[7,11,26–29]. These factors facilitate intestinal colonization by pathogenic facultative aerobes, such as *E. coli* K1, which can reach the bloodstream and the brain[7,30]. The K1 capsule has been identified in up to 80% of *E. coli* isolates responsible for neonatal meningitis[11,22]. The maternal gut microbiota can be a reservoir for *E. coli* K1, from which it can be vertically transmitted to the neonate during or after birth[6,22]. Eliminating encapsulated *E. coli* from the maternal gastrointestinal tract prior to birth could therefore decrease the risk of neonatal sepsis and meningitis. However, exposure to antibiotics during pregnancy and early life can have negative consequences on microbiome composition and the developing immune system of the neonate. Additionally, pathogenic *E. coli* are increasingly multi-drug resistant[31–34]. In contrast, bacteriophages (phages) producing tail fibers with endosialidase domains use the K1 capsule as a primary receptor to specifically infect and kill *E. coli* K1[35,36]. Importantly in this case, selection for phage resistance is an advantage as it implies the loss of the K1 capsule, a critical virulence factor[37–40].

Without the protective capsule, the immunogenic surface antigens of *E. coli* are exposed[15]. Therefore, we hypothesized that K1 specific phages could be combined with an oral vaccination scheme against the capsule-less strain. High-affinity intestinal secretory IgA (SIgA) would trap the capsule-less phage escapers via enchained growth[41]. To further increase the intestinal clearance of *E. coli* K1, we introduced a probiotic enabling vaccine-enhanced competition[42]. In this proof-of-principle, we used the probiotic *E. coli* Nissle1917 (K5:O6:H1), which has surface glycans and proteins that are not cross-reactive to the K1-specific phages or the SIgA induced by the vaccine used in this study[43]. This combined approach increased colonization resistance against *E. coli* K1 and was applied to pregnant dams in a murine vertical transmission model to delay neonatal gut colonization by *E. coli* K1 until weaning of the pups, thereby reducing the risk of neonatal meningitis.

Results

Phages can drive evolution of a capsule-less population with increased susceptibility to serum killing

We first tested whether phage-steering can drive loss of the K1 capsule. We used the clinical *E. coli* K1 strain LU_100[44] (Ec^{K1}), isolated from a neonatal case of sepsis in Switzerland. This isolate belongs to the serotype K1:O75:H5 and sequence type (ST) 1193, a worldwide emerging ST associated with sepsis and antibiotic resistance[45]. Using Ec^{K1} as host, three phages requiring the K1 capsule for infection were isolated from sewage water, $\Phi_{100\text{-}A}$, $\Phi_{NW\text{-}B1}$ and $\Phi_{NW\text{-}E1}$. Whole-genome sequencing revealed that the three phages belonged to the family *Autographiviridae*, closely related to two previously characterized K1 specific phages[35,46] (Supp. Fig. 1a). Spotting of the phages individually or combined at equal proportions in a cocktail ($K1\Phi_{cocktail}$) yielded plaques on lawns of Ec^{K1}, but not on the capsule knockout $Ec^{K1}\Delta kps$ or on *E. coli* expressing other capsule types (EcN^{K5} and Ec^{K23}, Supp. Fig. 1b-e). When Ec^{K1} was exposed to the phages in liquid medium, initial bacterial growth was followed by a reduction of the optical density (OD) due to lysis of the sensitive population (lysis phase, after roughly 2 hours). After this, a phage-resistant population quickly emerged and reached maximal OD within 12 hours (Fig. 1a). Colonies of the phage-resistant clones exhibited a translucent morphology on salt-free Lysogeny Broth (LB) agar. Capsule loss was confirmed in these clones by sequencing and capsule labelling with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by Ultra-performance liquid chromatography (UPLC) analysis[47] (Fig. 1b-d, Supp. Fig. 1f-g). Sequencing of seven phage-resistant clones revealed mutations in different genes within the capsule biosynthesis cluster, all leading to loss of the capsule (Supp. Fig. 1f-g, Supplementary Data 1 and 2)[48,49]. Four of the resistant clones had point or frame-shift mutations in the *neuC* gene (Supplementary Data 1 and 2) (Ec^{K1} *neuC**)[48]. NeuC catalyzes the formation of N-acetylmannosamine (ManNAc) and Uridine diphosphate (UDP) from UDP-N-acetylglucosamine (UDP-GlcNAc) as the first committed step in Cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac or CMP-Sialic acid) biosynthesis, an essential precursor for the K1 capsule[48]. Growth dynamics of constructed and natural mutants lacking the capsule ($Ec^{K1}\Delta kps$, $Ec^{K1}\Delta neuC$, Ec^{K1} *neuC**) showed no lysis phase in the presence of isolated phages or the cocktail (Supp. Fig. 2a-c). Accordingly, the phages could not replicate on strains lacking the capsule (Supp. Fig. 2d). Together these results demonstrated that the phages required the K1 capsule for infection and strongly selected for capsule-less mutants. The translucent colony morphology phenotype clearly associated with capsule loss was thereafter used to monitor the evolution of capsule-less mutants in a population by plating.

While phages can show exquisite selectivity, the fact that our phages all relied on the K1 capsule for infection suggested that we could target a broad range of K1-producing *E. coli* strains. To evaluate the host range of our phages, the individual phages and the $K1\Phi_{cocktail}$ were spotted on lawns of *E. coli* K1 isolates from various Sequence Types (ST) and expressing different O- and H-antigens (Ec^{K1_009}, Ec^{K1_054}, Ec^{K1_065}, Ec^{K1_068}, Ec^{K1_058}, Supp. Fig. 3a). While the individual phages showed variable infectivity, the $K1\Phi_{cocktail}$ could robustly infect the isolates as long as they produced the K1 capsule (Supp. Fig 3). This highlights the benefit of using a phage cocktail as opposed to individual phages. Individual bacterial strains may harbor phage defense systems, such as CRISPR-Cas[50], which can only be neutralized by some of the phages present in the cocktail[51]. Overnight growth curve analysis in presence of the $K1\Phi_{cocktail}$ revealed initial lysis phase and subsequent regrowth of a capsule-less population in all tested Ec^{K1} strains, including the most prevalent neonatal sepsis sequence type ST95 (Supp. Fig. 4).

In line with the known functions of the K1 capsule in protecting the bacteria against the innate immune system[21–24], hence increasing virulence[52], incubation of capsule-less Ec^{K1} mutants ($Ec^{K1}\Delta$*kps* and Ec^{K1} *neuC**) in human serum revealed increased susceptibility to serum killing compared to wild-type (Fig. 1e). Accordingly, the capsule-less *E. coli* mutants $Ec^{K1}\Delta$*kps* and $Ec^{K1}\Delta$*neuC* were also less virulent in a sepsis model compared to wild-type Ec^{K1} (Fig. 1f, Supp. Table 1). This demonstrated that phage selection could be leveraged to drive virulence attenuation in pathogenic *E. coli*. We then asked whether this holds true in a clinically relevant ecological context.

The main ecological niche of *E. coli* K1 is the large intestine, and invasive infections commonly arise from translocation of the patient's own resident *E. coli* to extra-intestinal sites[5,11,22]. To test the efficacy of phage-driven capsule loss in the intestinal context, ampicillin pretreated C57BL/6J mice with low complexity microbiota (LCM)[53] were infected orally with 10^8 Colony Forming Units (CFU) of Ec^{K1}, followed by 10^9 Plaque Forming Units (PFU) of the $K1\Phi_{cocktail}$ (Fig. 2a). In this model, Ec^{K1} robustly colonized the gut of wild-type mice without causing detectable intestinal inflammation, as measured by fecal Lipocalin-2 (LCN-2), a highly sensitive biomarker for intestinal inflammation[54] (Supp. Fig. 5a). In all mice treated with phages, we observed emergence of capsule-less mutants that reached fixation at the end of the experiment in all mice except three treated with $\Phi_{NW\text{-}B1}$ alone and one treated with $K1\Phi_{cocktail}$ (Fig. 2b, Supp. Fig 5b). There was no significant difference in fecal bacterial loads between phage-treated and control mice, suggesting that the open niche for *E. coli* in these mice is rapidly filled by capsule-less mutants selected for upon phage exposure (Fig. 2b-c, Supp. Fig. 5b-c). Phage titers diminished below detectable levels by day 6 post-

infection in most mice, following the trend of the increasing fraction of capsule-less Ec^{K1} (Fig. 2b,d, Supp. Fig. 5b,d).

Furthermore, we isolated capsule-less clones of Ec^{K1} from the feces. Sequencing of two clones revealed mutations in *emrR*, a transcriptional regulator of capsule biosynthesis[55] (Supp. Fig. 1f). Growth dynamic, phage replication assay and UPLC analysis revealed loss of capsule as well as abolished phage infection and the mutants were found to be susceptible to serum killing in line with published literature on the role of the K1 capsule[21–24] (Supp. Fig. 1f, Supp. Fig. 5e-j).

We inoculated LCM mice without antibiotic pretreatment with the distantly related *E. coli* K1 ST95 isolate Ec^{K1_058}, followed by the $K1\Phi_{cocktail}$ (Supp. Fig. 6a). In presence of phage, the percentage of capsule-less mutants increased significantly. The phenotypic detection of capsule mutants on salt-free LB agar was confirmed by sequencing isolated clones, which revealed large deletions spanning over the capsule biosynthesis gene cluster (Ec^{K1_058} *kps** 1 and Ec^{K1_058} *kps** 2 with 66 and 36 kb deletion respectively) (Supp. Fig. 6b, Supplementary Data 1 and 2). Again, the overall bacterial load was not significantly different between groups and we observed a consistent maintenance of phages in the gut, in line with higher frequency of capsulated clones than when Ec^{K1} was exposed to the same phage cocktail *in vivo* (Supp. Fig. 6c-d, Fig. 2c-d).

Combination of phage therapy and oral vaccination increases colonization resistance and intestinal clearing of Ec^{K1}

Reversion of capsule loss could happen relatively frequently in the cases of point mutations and horizontal gene transfer could repair large deletions[56]. Therefore, it is important to exclude the capsule mutants from the gut[57]. In the intestinal environment, secretory Immunoglobulin A (SIgA) elicited through oral vaccination can drive competitive exclusion of specific strains[42,58]. While the K1 capsule is poorly immunogenic, the underlying surface antigens can induce a strong SIgA response upon vaccination with peracetic acid (PA)-killed whole cells[15,58,59].

We observed that after four oral doses of PA-killed $Ec^{K1}\Delta kps$ (PA-$Ec^{K1}\Delta kps$), mice produced an SIgA response specific to non-capsular surface antigens[42,58] that strongly selected for Ec^{K1} and against $Ec^{K1}\Delta kps$ on competitive colonization of the gut, due to shielding of SIgA-reactive surface antigens by the capsule in Ec^{K1} (Supp. Fig. 7).

We reasoned that combining phages against Ec^{K1} with oral vaccination targeting Ec^{K1}Δ*kps* might suppress gut colonization by both Ec^{K1} and capsule-less mutants, which should mitigate the risk of capsule restoration in capsule-less mutants. Moreover, niche competition is essential for vaccine-enhanced intestinal clearance[42]. LCM mice are devoid of competing *E. coli* strains and we therefore introduced the probiotic *E. coli* Nissle1917 (EcN, serotype K5:O6:H1) as a non-cross-reactive niche competitor to enable intestinal clearance of the pathogen (Fig. 3a, Supp. Fig. 1d). This recapitulated the situation in humans where several *E. coli* strains generally share the intestinal niche[60,61].

Without EcN, the Ec^{K1} fecal loads were comparable to the naïve group, reflecting the carrying capacity and the lack of natural niche competitors in the LCM mice[53] (Supp. Fig. 8a-c). Oral vaccination with PA-Ec^{K1}Δ*kps* induced production of SIgA directed against Ec^{K1}Δ*kps* in the intestine but not against EcN (Fig. 3b, Supp. Fig. 8d). As expected, this SIgA did not bind strongly to the surface of wildtype Ec^{K1} due to shielding by the K1 capsule, and had no significant effect on the competition between wild-type Ec^{K1} and EcN (Fig. 3c, Supp. Fig. 7e). In contrast, the Ec^{K1} load was reduced 100-fold with oral phage treatment alone (Fig. 3d). Oral vaccination and phage treatment exhibited additive effects on reducing the average Ec^{K1} levels 1000-fold compared to the control mice (Fig. 3d). In six vaccinated and phage treated mice, no Ec^{K1} could be detected 6 days post infection, i.e. complete clearance from the gut was achieved. There was no significant difference in the fecal loads of EcN between the treatment groups (Supp. Fig. 8e). Correspondingly, the ratio of Ec^{K1} to EcN was significantly reduced the last three days in the vaccine and phage treated group compared to all other groups, largely reflecting the change in Ec^{K1} loads (Fig. 3c-d). Phage titers were reduced over the course of the experiment (Supp. Fig. 8f). In all animals where phenotypically K1-positive Ec^{K1} could be detected, phage titers remained at around 10^4 PFU/g_{feces}. In all animals where 100% of fecal Ec^{K1} lost the capsule, or where the entire Ec^{K1} population was eliminated, phage titers were undetectable (Supp. Fig. 8f-g). This demonstrated that phage therapy and vaccine-induced mucosal immunity can have additive effects that results in major loss of fitness for the targeted *E. coli* K1 strain, in some cases allowing complete exclusion of this opportunistic pathogen from the gut.

Combined oral vaccination and phage treatment in pregnant mice prevent vertical transmission of *E. coli* K1

While vertical transmission of maternal gut microbes is generally considered beneficial for neonatal health and development[62], *E. coli* K1 transmission from colonized mothers can cause sepsis or meningitis in children after birth[7,11]. Therefore, we tested whether our intervention could sufficiently reduce intestinal load of Ec^{K1} in the gut of pregnant mice to prevent transmission to neonates. In order to make this model

more translationally relevant, we carried out all manipulations without antibiotic pre-treatment, i.e. in the context of an unperturbed intestinal environment. Female mice were orally vaccinated against Ec^{K1}Δ*kps* or mock-vaccinated with PBS once a week for three weeks prior to mating. The females received the fourth, final dose of the vaccine during pregnancy (one week after mating) to increase chance of IgA transmission to pups via milk. One day after the last vaccine dose, the pregnant females were first colonized with EcN and 24 hours later with Ec^{K1}, before receiving phage treatment or exhausted LB as control (Fig. 4a). Vaccination and colonization were done by voluntary feeding, to reduce stress during pregnancy and to investigate the efficiency of our approach in a more realistic model. Daily fecal sampling from the pregnant females revealed plateauing Ec^{K1} colonization at 10^8 CFU/g feces in all control groups and reduced Ec^{K1} loads by 10- to 100-fold in the vaccinated and phage treated group, consistent with previous experiments (Fig. 3c-d, Fig. 4b-c). In this setting, the fecal Ec^{K1} bacterial loads were initially reduced by phage treatment alone, followed by rapid re-population with the emerging capsule-less mutants (Fig. 4b-c, Supp. Fig. 9a), confirming the benefit of combining phage treatment with oral vaccination compared to phage treatment or vaccination alone. Consistent with previous experiments, the fecal levels of EcN and the phages did not differ between the groups (Supp. Fig. 9b-c). The gut microbiota was not cleared with antibiotics beforehand. Therefore, the mice harbored a microbiota conferring higher colonization resistance. These experiments confirmed that our strategy also worked at reduced *E. coli* colonization levels comparable to the average population size of *Enterobacteriaceae* observed in humans, i.e., 10^8 bacteria/g of feces[63].

At day of life (D.O.L.) 10, the entire or a part of the litter was euthanized to quantify the intestinal *E. coli* colonization levels in the pups. We observed transmission of Ec^{K1} without any treatment (in 11/13 pups (80%)) (Fig. 4d). Vaccination alone had no effect on transmission (20/22 pups (90%) were colonized) (Fig. 4d), consistent with the K1 capsule blocking the O-antigen-mediated IgA binding (Supp. Fig. 7e). In the phage-treated group, 63% (12/19) of the pups were colonized with Ec^{K1}, however all pups in this group were colonized by capsule-less mutants, reflecting the fixation of a capsule-less population in the mothers (Fig. 4d, Supp. Fig. 9a,d-n, Supplementary Data 2). In contrast, only 23% (3/13) of pups from vaccinated and phage treated mothers were colonized Ec^{K1} at D.O.L. 10 (Fig. 4d). Reflecting the exclusion of Ec^{K1}, a larger fraction of pups from vaccinated and phage treated mothers was colonized by probiotic EcN compared to control groups (Supp. Fig. 10a).

To investigate the timing of colonization of the pups, the remaining part of or entire litters were swabbed carefully in the rectum with a pipette tip daily until D.O.L. 21. Enrichment cultures thereafter revealed that

most pups born to vaccinated and phage treated mothers were protected from Ec^{K1} colonization until D.O.L. 16, when at least 60% of pups in all control groups were already colonized by Ec^{K1} (Fig. 4e, Supp. Fig. 10b-c). At this time, roughly 22 days post inoculation, the unvaccinated females produced sufficient IgA for transfer to the pups via the milk, as can be seen in the IgA titers from the small intestinal and stomach content of the pups (Supp. Fig. 10d-e). Although long-term colonization was sufficient to produce an anti-*E. coli* IgA response[64,65] (Supp. Fig. 10e-f), it did not suppress *E. coli* transmission to the pups even in the phage-treated group, where the population was completely capsule-less and the surface antigens exposed (Fig. 4d-e, Supp. Fig. 9d). This gives some insight into the mechanisms underlying protection i.e., the maternally transmitted IgA was not protecting the pups against Ec^{K1} transmission. Instead, the reduction of the overall levels of Ec^{K1} gut colonization in the mother by combined treatments was necessary to decrease the rate of transmission of Ec^{K1} from mothers to pups. In addition, as all dams developed O75-antigen specific SIgA by the end of the experiment, we noted that the function of vaccine-induced SIgA was superior to naturally developed SIgA in suppressing gut colonization levels.

Discussion

The K1 capsule is a key virulence factor in *E. coli* strains causing neonatal sepsis and meningitis, especially in preterm and low birth weight infants[6,7,11]. While screening and intra-partum administration of antibiotics has significantly reduced the burden of neonatal sepsis caused by group B Streptococcus (GBS), there are currently no successful strategies to prevent disease caused by *E. coli*[66,67]. In addition, relying on antibiotics for treatment becomes increasingly unattractive, due to a high incidence of extended-spectrum beta-lactamase-mediated resistance and long-term negative consequences of a disrupted gut microbiota in early life[31–33,66]. Alternatively, administration of probiotics or synbiotics to neonates has been evaluated as a preventive strategy against sepsis, where some report success[68,69] and others no effect[70]. Here, we directly target the K1 capsule with phages that select for capsule-less mutants in various clinical neonatal sepsis isolates of *E. coli* K1 *in vitro* and *in vivo*.

As observed in previous studies assessing K1-targeting phages to treat infections by *E. coli* K1 strains[38,40], capsule mutants were selected when phages were given orally to target *E. coli* K1 in the intestinal tract of mice. From *in vitro* experiments, seven capsule-less isolates were sequenced and mutations were only detected in the capsule biosynthesis gene cluster (*neuC*, *neuD*, *neuB*, Supplementary Data 1 and 2). From colonization experiments, we sequenced 10 capsule-less clones and identified mutations in the capsule biosynthesis gene cluster as well as in the regulator of these genes in (i.e. *emrR*) or large deletions (>10kb) of the capsule locus. No other relevant genomic mutations were detected (Supplementary Data 2). These

differences between *in vitro* and *in vivo* conditions in our study could be a matter of sample size or specific factors in the gut may select for different types of mutants. Our dataset did not allow us to draw any conclusions. Nevertheless, the clear pattern was that all phage resistant clones had mutations that resulted in loss of capsule, suggesting that this was the only evolutionary outcome of the phage treatment.

The evolved capsule-less mutants were both more susceptible to serum killing and unable to cause disease when injected subcutaneously in mice (Fig. 1e-f), confirming the role of the K1 capsule in *E. coli* pathogenesis[8,9,52]. Steering the evolution of attenuated bacterial populations with phages has previously been highlighted as a potential intervention to improve clinical outcome by leveraging the rapid evolution of phage resistance[71]. Here we show that such a strategy could work in the ecological context of the intestinal microbiome. We were able to drive the fixation of capsule mutants in populations of *E. coli* K1 reaching loads comparable to the load of *Enterobacteriaceae* in humans[63]. Nevertheless, interindividual variations can be substantial in humans and our approach may reach its limits when *E. coli* intestinal loads are too small to be targeted by bacteriophages.

As a second line of defense, we invoked the host's mucosal immune system to eliminate the evolved capsule-less mutants with intestinal SIgA elicited by oral vaccination against $Ec^{K1}\Delta kps$ (Fig. 3). The final essential part to increase colonization resistance against *E. coli* K1 was to introduce the niche competitor EcN, which benefits from phage- and SIgA-mediated suppression of *E.* coli K1 fitness to take over the niche and further reduce the bacterial load of Ec^{K1} and derivatives via vaccine-enhanced competition[42] (Fig. 3). Each part of this combined approach provided an additive effect to increase colonization resistance against *E. coli* K1.

Furthermore, the strategy could be translated into a vertical transmission model where the pups were protected from *E. coli* K1 colonization during the first vulnerable days of life, if the mother received both vaccination and phage treatment (Fig. 4). To our knowledge, this was the first demonstration of specific prevention of transmission of a neonatal sepsis-causing *E. coli* with important implications for neonatology[7,26]. Phage treatment alone did not provide protection against vertical transmission during early life, however the cells transmitted were attenuated capsule-less clones unable to survive systemically, which may be sufficient to prevent disease (Supp. Fig. 9d). On the other hand, there is a risk of capsule restoration when the evolutionary pressure exerted by the phage is lifted, as the capsule can confer protection when colonizing the gut[15,39,57,72]. In our setting, oral vaccination against the capsule-deficient *E. coli* alone did not protect from transmission of *E. coli* K1, in line with the K1 capsule effectively blocking IgA-O-antigen interactions (Supp. Fig. 7e). Sereme et al., on the other hand, recently proposed

intraperitoneal vaccination of pregnant females with a live attenuated *E. coli* K1, where the transmitted antibodies protected the pups against systemic infection, but not colonization per se, suggesting there may be an additional benefit to capsule-independent antibody responses in invasive disease[73].

The limitations of the study include a large variability in Ec^{K1} colonization levels between mice in the vaccinated and phage-treated group: some mice were colonized at 10^7 CFU/g of feces, while in others, no Ec^{K1} were detected. (Fig. 3d). This could be explained by differences in the pace at which the competing microbiota repopulated the gut after antibiotic treatment[74]. The outcome of vaccination (antibody affinity, specificity and overall titres) could also vary between animals, eliciting heterogenous selective pressure from IgA-mediated enchained growth against capsule mutants. Moreover, EcN was likely far from the perfect competitor: no attempt was made to predict the metabolic overlap between EcN and our *E. coli* K1 strains of interest, rather EcN was chosen as an established probiotic. Considerable further work will be required to identify strains or cocktails of strains that robustly compete for the metabolic niche of the opportunistic pathogen in a wide range of microbiota backgrounds[42,75]. EcN was nevertheless competitive enough to exclude Ec^{K1} when specifically targeted by phages and SIgA in some animals. We also cannot exclude that EcN contributed to the exclusion of *E. coli* K1 via the production of the genotoxic colibactin[76]. Therefore, although we have demonstrated the validity of the concept that the combination of these three interventions may be sufficient to eliminate the chosen strain of *E. coli* K1 from the gut, further work on the underlying mechanisms and development of the treatment will be needed to make this approach truly robust.

Another limitation of our study is that for reasons related to animal welfare, we employed strains of *E. coli* K1 that do not cause overt disease in C57BL/6 mice. This meant we could not assess the protective efficacy of our interventions against invasive disease. Nevertheless, invasion cannot occur without exposure, and typically requires gut colonization. Therefore, successfully preventing gut colonization and reducing spread from gut reservoirs of infection is highly likely to be an effective intervention.

For clinical application, we also need to address the issue of the specificity of the intervention in relation to the strain. In this proof-of-principle, we have focused mainly on a neonatal sepsis isolate of *E. coli* expressing the K1 capsule (LU100). K1 is the most prevalent capsule type in *E. coli*-associated neonatal meningitis (80%), and is common in other types of extraintestinal infections in children and adults (25% ExPEC)[11,12,22,77,78]. Our phage cocktail successfully drove the loss of the K1 capsule, both *in vitro* and in mice, in two strains belonging to distinct sequence types: ST1193, an emerging cause of community-acquired and nosocomial sepsis, and ST95 prevalent in neonatal meningitis[79]. However, unlike the similar results

seen *in vitro*, we did not observe the complete fixation of K1 capsule mutants in the Ec^{K1_058} strain ST95 exposed to phages in mice (Supp. Fig. 6). This suggested that the genetic background of the *E. coli* strain could influences the outcome of the phage-bacteria interaction within the intestinal tract. It is unclear whether this was specific to the sequence type, or could result from strain-level diversity. Consequently, phage cocktails must be optimized to account for a potentially very broad range of strain-specific factors, with further research on a much wider panel of *E. coli* strains needed to understand the exact determinants. Furthermore, no antibiotics were used to clear the microbiota before colonizing with Ec^{K1_058}, which influences the carrying capacity and correspondingly the probability of phage-bacteria interaction.

When associating phages with vaccination, the diversity of O antigens within and between sequence types must be taken into account[80,81]. Combining multiple strains in an inactivated whole-cell oral vaccine is relatively simple[58] and is already employed in the oral cholera vaccine. For example, including *E. coli* O1, O2, O18 and O75 in the vaccine composition would cover more than 60% of the O-antigen variations in *E. coli* K1 causing neonatal meningitis[80,81]. Therefore, it should be possible to extend this approach to any clinically relevant sequence type or the second most frequent capsule-type, K5, by including additional bacteriophages and enriched vaccine compositions[76,77]. In principle, the concept of combining bacteriophages, oral vaccination and live-bacterial competitors should also be effective against more distantly-related but clinically relevant strains or species such as *Klebsiella*[82,83].

The complexity of the phage/vaccine cocktail needs to be balanced against the potential clinical benefit and the cost of licensing a multi-component product. It should be noted that the treatment of neonatal sepsis or meningitis represents a considerable financial burden on the health care system; in 2021, this burden was estimated at over $200 million in the United States alone[84–86]. In addition, with the rising threat of antimicrobial resistance, treating neonatal bacterial infections becomes more challenging and new preventive strategies are essential[6,66]. Screening programs to identify and treat mothers during pregnancy are already established for Group B Streptococci[87] and we could envision a similar approach here, suppressing the opportunistic pathogen load in mothers during pregnancy to prevent early neonatal exposure. It is worth noting that the inactivated oral vaccine, bacteriophages and probiotic were all administered orally and could be combined into a single dose – the vaccine was very similar to oral cholera vaccines that are licensed and successfully deployed in low-resource settings[88], suggesting that our approach could be relevant globally. Clinical production of bacteriophages remains a field in its infancy, with personalized “compassionate use” treatments relatively costly. However, gut-adapted

bacteriophages are extremely robust organisms that can be stored under a range of conditions and the main cost-barrier currently is determined by lack of scale-up production and established translational pipelines[89]. Oral probiotics are low-cost and generally considered safe. Therefore, we believe that the combined vaccine and phage cocktail represent a viable and targeted approach that could reduce the emotional and financial cost of treating neonatal sepsis or meningitis. In addition, reducing the carriage rate of opportunistic pathogens could reduce the overall burden of infections caused by these strains, as well as antibiotics needed to treat these infections, hence lowering the resistance risk.

In conclusion, we have successfully developed a strategy to increase resistance to colonization by pathogenic *E. coli*, which is of major clinical interest. This strategy can be applied to protect newborns against colonization by *E. coli* K1 early in life, likely through the suppression of colonization in the pregnant mother and, consequently, the reduction of the risk of transmission during parturition. **Methods**

Ethics statement

This research complies with ethics regulations. All mouse colonization experiments were performed in accordance with Swiss Federal regulations approved by the Commission for Animal Experimentation of the Kanton Zürich (licenses 120/2019 and 066/2021; Kantonales Veterinäramt Zürich, Switzerland, 33580; Kantonales Veterinärarmt Basel, Switzerland)[54].

The mouse sepsis experimental protocol (APAFIS#4948) was approved by the French Ministry of Research and by the Ethical Committee for Animal Experiments, CEEA-121, Comité d'éthique Paris-Nord.

Culture conditions

The *E*. *coli* K1 isolates originate from the Swiss Child Sepsis Cohort[44] (Supplementary Data 1). Bacteria were cultivated in LB (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) medium at 37°C with shaking at 180 or 200 rpm (Infors HT Multitron Standard) unless otherwise stated. Media was supplemented with appropriate antibiotics (100 µg/ml ampicillin (AppliChem); 50 µg/ml apramycin (Merck); 6 µg/ml chloramphenicol (AppliChem); 50 µg/ml streptomycin (AppliChem)).

All dilutions were prepared in phosphate buffer saline (PBS).

Phage isolation from sewage water

Inlet sewage water from Emmenbrücke (LU, Switzerland) and Basel (BS, Switzerland) was filtered (0.45 µM), concentrated by adding $ZnCl_2$ to a final concentration of 40 mM and pelleted by centrifugation. The

pellet was resuspended in 1 ml Saline-Magnesium (SM) buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-Cl pH 7.5) and 150 µl or 600 µl were mixed with 250 µl Ec^{K1} overnight culture, incubated at room temperature for 15 minutes without agitation and mixed with 10 ml warm of top agar (0.5% agar, 1 mM $MgSO_4$, 1 mM $CaCl_2$) and poured onto LB agar square plates (12 cm x 12 cm (Greiner)). After drying, plates were incubated at 37°C overnight. Morphologically different plaques were transferred into 1 ml LB. Dilutions of phage lysates were spotted onto a lawn of Ec^{K1}. This process was repeated three times to purify the obtained phages.

Phage stocks were prepared by adding phages to 10 ml LB with Ec^{K1} grown to OD_{600} 0.1 at multiplicity of infection (M.O.I.) 0.1 and incubating with shaking at 37°C overnight. Simultaneously 1000 PFU of phages were added to 10 ml warm top agar with 100 µl overnight culture of Ec^{K1}, poured onto an LB agar plate and incubated at 37°C overnight. Using a 50 ml conical plastic tube (TPP, Switzerland), the lysis zones in the top agar were scraped off and mixed with the overnight culture containing phages. The mix was pelleted and the supernatant was sterilized by filtration.

Phage DNA was isolated from 1 mL of DNase and RNase treated lysates (10^{10} PFU/mL), using the Norgen Phage DNA Isolation Kit (cat. 46850). Phage genomes were sequenced by SeqCenter (https://www.seqcenter.com/) using Illumina technology. Reads were assembled with Unicycle v0.5.1[90] and the resulting assembly are available on Genbank (Accession numbers: $\Phi_{100\text{-}A}$; PQ476009, $\Phi_{NW\text{-}E1}$; PQ476010, $\Phi_{NW\text{-}B1}$; PQ476011)(Supp. Table 2).

Plaque assay

In vitro samples containing phages were sterilized using 0.22 µm filters. Fecal samples were sterilized by adding 20 µL of chloroform to 200 µL of sample, vortexed for 15 seconds and centrifuged for 3 minutes at 17'000x *g*. 10 ml warm top agar were mixed with 200 µl liquid culture of the reporter strain and poured onto LB agar plates (1.5% agar). Unless otherwise stated, 10 µl of dilutions of phage samples were spotted onto the bacterial lawn and the plates were incubated overnight at 37°C.

Construction of *E. coli* mutants

Mutants were constructed by lambda red recombination[91]. Primers are listed in Table 1. Electrocompetent cells were prepared by inoculating 2 ml salt-free LB (10 g/L tryptone, 5 g/L yeast extract) supplemented with appropriate antibiotics with the parental strain and incubated overnight at 37°C or at 30°C when temperature-sensitive plasmid such as pKD46 were used [92]. Plasmids are listed in Table 2. The overnight culture was diluted 1:100 in fresh salt-free LB supplemented with appropriate antibiotics and incubated at

37°C. At OD_{600} = 0.3, EDTA was added to a final concentration of 5 µM and further grown until OD_{600} reached 1. When the parental strain contained pKD46, the day culture was additionally supplemented with 0.2% L-arabinose to induce expression of the λred system and grown at 30°C. The cells were pelleted and washed twice with ice-cold sterile water.

Phage replication assay

20 µl of *E. coli* cultures grown in LB supplemented with 100 µg/ml ampicillin (AppliChem) overnight at 37°C and 200 rpm was diluted in 2 ml of LB in a 50 ml conical plastic tube (TPP) and incubated 2 hours at 37°C and 200 rpm. 10^3 PFU of phages were added to each tube and further incubated for 4 hours. 1 ml was pelleted and either filtered (0.22 µm filter) or treated with 20 µl chloroform for sterilization. Phage titers were determined by plaque assay.

Growth curves

E. coli overnight cultures were diluted 1:100 in 200 µl LB in a transparent 96 well plate (TPP) and infected with phages at M.O.I. 0.01. The plates were incubated with lid overnight at 37°C with orbital shaking in an Infinite M Plex (Tecan) or a Synergy H4 (Biotek) plate reader with OD_{600} measurements every 10 minutes.

Ultra-performance liquid chromatography (UPLC) quantification of sialic acid

1 ml *E. coli* overnight culture was pelleted, washed once in 1 ml MilliQ H_2O and resuspended in 40 µl 20 mM trifluoroacetic acid (TFA). 10 µl cell sample was mixed with 1,2-diamino-4,5-methyleneoxybenzene (DMB) labelling solution (10 mM DMB, 500 mM beta-mercaptoethanol, 20 mM sodium dithionite and 20 mM trifluoracetic acid (TFA)) and incubated 2-3 hours at 45°C. To avoid lactonization of sialic acid, each sample was supplemented with 20 µl 140 mM NaOH and further incubated at 45°C for 30 minutes. 200 µl of 20 mM Tris (pH 7.5) was added to the samples and they were centrifuged at 20'000x *g* for 20 minutes. 80 µl of the supernatant was injected on a DNAPac (ThermoFisher Scientific) column and separated in a gradient of NaCl (0-1 M) in 20 mM Tris pH 7.5 with a flow rate of 1.2 ml/min. Column oven was set to 35°C. Fluorescence intensity was measured after excitation at 373 nm and emission at 448 nm. The chromatograms were analysed in Chromeleon 7.2 (Thermo Fisher Scientific).

Serum killing assay

Heat-inactivated serum was prepared by heating at 56°C for 30 minutes[24]. Three independent cultures in exponential phase were diluted to $2*10^5$ CFU/ml in 200 µl of active or heat-inactivated human serum

(H4522, Merck) and incubated at 37°C for 1 hour. At time point 0, 30 minutes and 60 minutes, a 20 µl sample was taken and dilutions were plated on LB agar for assessing bacterial viability.

Murine model of gut colonization

Low complexity microbiota (LCM)[53] C57BL/6J mice were used in all gut colonization experiments. Mice were bred and housed in individually ventilated cages with a 12 hour light/dark cycle at the ETH Phenomics Center (EPIC, RCHCI), ETH Zürich or in ISOcages at WRO Basel and were fed a standard chow diet. All mice included in experiments were 4 weeks or older and objectively healthy as determined by routine health checks. Wherever possible, an equal number of males and females was used in each group.

In all murine gut colonization experiments, Ec^{K1} with an apramycin cassette inserted in *glms*[95] (Ec^{K1} Tn7::*aac3_VI*) for selection and EcN transformed with pBAD24[93] to allow antibiotic pretreatment with ampicillin, were used. At endpoint, mice were euthanized with CO_2 and cardiac heart puncture. Blood from the heart was collected in serum tubes (Sarstedt) and centrifuged at 10000x *g* for 5 minutes to obtain serum, heat inactivated at 56°C for 30 minutes and stored at -20°C until further analysis. Intestinal lavages were collected in a 2 ml Eppendorf tube by flushing the small intestine with 1 ml PBS using a cannula. Bacterial and fecal particles were cleared from the intestinal lavage by centrifugation for 10 minutes at 17'000x *g* and stored at -20°C until further analysis.

Murine model of sepsis

The virulence of *E. coli* K1 and derivative strains was assessed using a murine model of sepsis[96]. 4 week old (14–16 g) female OF1 mice from Charles River (L'Arbresle, France) were inoculated with 10^8 *E. coli* cells subcutaneously in the neck and monitored for six days. Housing conditions for these mice were in agreement with the French law, with dark/light cycle, and constant ambient temperature (21°C + /− 2°C) and humidity (50% +/− 10%). The bacteria were cultivated overnight in LB then washed and re-suspended in 200 µl of physiological water before inoculation. The mice were inoculated in blind experiments by the zootechnician who ignores the status of the strains.

Time to death was recorded during the following 7 days. Mice surviving more than 7 days were considered cured and sacrificed[97]. In each experiment, the *E. coli* CFT073 strain was used as a positive control, killing all the inoculated mice, whereas the *E. coli* K-12 MG1655 strain was used as a negative control for which all the inoculated mice survive[96].

Sequencing

Bacterial DNA was purified using Quick-DNA Miniprep Plus Kit (Zymo) or NucleoBond with AXG 20 columns and Buffer Set III (Machery-Nagel). Phages and bacterial mutants (MDBZ1109, MDBZ1346, MDBZ1646-1647) were sequenced using Illumina Next-Generation Sequencing (NGS), the reads were assembled using Unicycler[90] and annotated using Prokka[98]. Bacterial mutant reads were mapped on the reference genome using BWA-MEM[99] and variants were called using LoFreq[100]. The Galaxy[101] platform was used for analysis. Oxford Nanopore MinION long-read sequencing was used for for bacterial isolates and mutants (MDBZ1877-1879, LLETH_278, LLETH_277, LLETH_275, LLETH_271, LLETH_283, LLETH_273, LLETH_284, LLETH_279, ESETH_C73, ESETH_C70, ESETH_C60, ESETH_C72, ESETH_C61, ESETH_C58, LLETH_313 and LLETH_314) was performed in-house at the Biozentrum, using the MinION Flow Cells R10 and the Rapid Barcoding Kit 24 V14 (Oxford Nanopore), or at ETH Zurich, using a MinION FLO-MIN114 Flow Cell (MinION Mk1C device, 72 h) and the Rapid Barcoding Kit (SQK-RBK114) . Basecalling and demultiplexing was performed with Guppy (Oxford Nanopore). Quality control was performed on raw reads using bbmap v38.87[102] with a trimming quality threshold of 14 and a minimum read length of 45 bp. Reads were mapped using the evo genome analysis pipeline (https://github.com/mmolari/evo-genome-analysis) or using Minimap2[103]. For ESETH_C73, ESETH_C70, ESETH_C60, ESETH_C72, ESETH_C61, ESETH_C58, LLETH_313 and LLETH_314, high-quality reads were assembled or Flye v2.9[104] with the “nano-raw” option for ONT (“pass”) reads and assembled genomes were polished using Pilon v1.23[105]. All tools were run with default parameters unless otherwise specified.

Strain ESETH_C73 was sequenced using both Illumina and Nanopore technologies, assembled and annotated by SeqCenter, using the Small Nanopore Combo service (https://www.seqcenter.com/services/). Sequences are freely accessible on the NCBI server with the Bioproject number PRJNA1402325.

Single infection with *E. coli* and phages in the murine model of gut colonization

When stated, the mice were orally treated with 20 mg ampicillin (AppliChem) by oral gavage 24 hours before colonization. *E. coli* was grown in 2 ml LB supplemented with 100 µg/ml ampicillin for Ec^{K1}, and no supplement for Ec^{K1_058} overnight at 37°C, 200 rpm, then diluted 1:20 in 2 ml fresh LB and cultivated for another 4 hours. The day culture was pelleted and washed twice with sterile PBS. The mice were orally infected with 10^8 CFU of *E. coli* by voluntary feeding on Zwieback (Migros) and after 30 minutes with 10^9

PFU of phages on Zwieback. Feces were collected daily and homogenized in 1 ml PBS by bead beating (3 mm steel or 2x 2 mm glass ball, 25 Hz for 3 min in a TissueLyser (Qiagen)). Bacteria were quantified by selective plating on LB agar with or without salt supplemented with appropriate antibiotics. Phage titer was determined by plaque assay. At endpoint, bacterial load in cecum and feces was determined as described above.

Competitions in the murine model of gut colonization

The mice were orally treated with 20 mg ampicillin (AppliChem) by oral gavage 24 hours before colonization. Ec^{K1} and Ec^{K1}Δ*kps* (Ec^{K1} *kps*::*cat*) were grown overnight in 2 ml LB supplemented with 100 µg/ml ampicillin at 37°C, 200 rpm and then diluted 1:20 in 2 ml fresh LB and cultivated for another 4 hours. The day cultures were pelleted and washed twice with sterile PBS. The cultures were mixed 1:1 and 10^8 CFU of the mixtures were provided to the mice by voluntary feeding on Zwieback (Migros). The initial ratio was estimated by selective plating. Feces were collected and treated as described above. Bacteria were quantified by selective plating on LB agar with or without salt supplemented with appropriate antibiotics. At endpoint, bacterial load in cecum and feces was determined as described above.

Vaccinations

The Ec^{K1}Δ*kps* Peracetic acid-killed (PA-killed) vaccine was prepared as described[59]. In short, Ec^{K1}Δ*kps* was grown overnight in 2 L LB at 37°C, 180 rpm shaking. The following day, the culture was centrifuged at 10'000x *g* for 10 minutes and the pellet was washed once with 50 ml PBS before incubating the cells in PBS 0.4% peracetic acid (Sigma-Aldrich) for 1 hour. The PA-killed cells were then washed once with 50 ml 10X PBS followed by three washes with 1X PBS before finally resuspending in maximum 15 ml. 10^{10}-10^{11} cells were then orally fed to the mice by pipetting the solution on Zwieback (Migros). Vaccinations were carried out once a week for 4 weeks. Mock-vaccinations were carried out by pipetting 100 µl PBS on Zwieback.

Murine gut colonization with niche competitor and Ec^{K1}, and phage treatment

Mice were orally pre-treated with 20 mg ampicillin by oral gavage 24 hours before oral infection with EcN followed by Ec^{K1} infection 24 hours later. Bacterial strains were cultivated overnight in 2 ml LB at 37°C, 200 rpm. Day cultures were prepared by diluting the overnight culture 1:20 in 2 ml fresh LB and cultivating further for 4 hours at 37°C, 200 rpm. 1 ml cells were pelleted and washed twice with 1 ml sterile PBS. 10^8 CFU of bacteria were provided to the mice by voluntary feeding on Zwieback (Migros, Switzerland). 30

minutes after infection with Ec^{K1}, 10^9 PFU of $K1\Phi_{cocktail}$ or 100 µl PBS were provided to the mice on Zwieback (Migros).

Feces were collected daily and homogenized in 1 ml PBS by bead beating (3 mm steel or 2x 2 mm glass beads, 25 Hz for 3 min in a TissueLyser (Qiagen)). Bacteria were quantified by selective plating on LB agar with 50 µg/ml streptomycin and LB agar without salt supplemented with 50 µg/ml apramycin. Phage titer was determined by plaque assay. At endpoint, intestinal lavages were collected and bacterial loads in cecum and feces were determined as described above.

Vertical transmission

5-6 weeks old female LCM mice were orally vaccinated with the Ec^{K1}Δ*kps* PA-killed vaccine by voluntary feeding on Zwieback weekly for four weeks. One male was introduced in a cage of two females after three weeks of vaccination (age of females: 7-8 weeks) and removed two weeks later. One day after the last vaccination, 10^8 CFU of EcN were prepared as described above and orally provided to the mice on Zwieback followed by 10^8 CFU of Ec^{K1} 24 hours later. 30 minutes after Ec^{K1} infection, 10^9 PFU of $K1\Phi_{cocktail}$ were provided to the mice on Zwieback.

Feces were sampled daily for 9 days and processed as described above. Approximately 12 days post infection, pups were born. 10 days post birth, a part of the litter was euthanized by decapitation. Blood was collected in serum tubes (Sarstedt), centrifuged at 10000x *g* for 5 min to obtain serum, heat inactivated at 56°C for 30 minutes and stored at -20°C until further analysis. Feces and cecum content were collected and processed as described above. The stomach and small intestine were collected and homogenized in 1 ml sterile PBS by bead beating (3 mm steel bead, 25 Hz for 3 min).

From 10-21 days post birth, feces from females were collected daily and processed as described above. Samples from pups were obtained by rectal swabbing using a sterile pipette tip which was placed in 1 ml LB for overnight enrichment at 37°C, 180 rpm. The overnight cultures were diluted and plated selectively on LB agar with 50 µg/ml streptomycin and LB no salt with 50 µg/ml apramycin for qualitative identification of bacterial strains. Detection of phages was done with plaque assay as previously described. At endpoint, mice were euthanized with CO_2 and cardiac heart puncture. Blood from the heart was collected in serum tubes and processed as described above. Intestinal lavage was collected by flushing the small intestine with 1 ml PBS using a cannula. Feces and cecum content were collected, and process as described above. Mesenteric lymph nodes were homogenized in 1 ml PBS by bead beating (3 mm steel ball, 25 Hz for 3 min) and bacteria quantified by selective plating on LB agar with or without salt.

Analysis of antibody titers by bacterial flow cytometry

Antibody titers in mouse intestinal lavages were measured by flow cytometry[106]. In short, intestinal lavages and blood were collected as described above. Bacterial targets were grown overnight in filtered LB (0.22 µm filters), then centrifuged 3 minutes at 7000x *g*. The pellet was washed twice with 0.22 µm-filtered PBS/0.02% azide/1% BSA. After thawing, the intestinal lavages were centrifuged at 17'000x *g* for 10 minutes and the supernatants were diluted in 96-well plates with conical bottoms (TPP). 10^5 washed cells were added to each well and incubated for 1 hour on ice, followed by three washes with 150 µl 0.22 µm-filtered PBS/0.02% azide/1% BSA and centrifugation at 7000x g for 5 minutes. Pellets were resuspended in 25 µl of 0.22 µm-filtered PBS containing monoclonal Brilliant Violet 421 Rat Anti-Mouse IgA (BD Bioscience, 2 µg/ml, 743293, AB_2741405) for detection of intestinal IgA. After 1 hour of incubation, cells were washed as described above but with 150 µl filtered PBS (0.22 µm) and resuspended in 100 µl PBS. Flow cytometry acquisition was performed using a Beckman Coulter Cytoflex S with SSC and FSC parameters in logarithmic scale. Data was analysed with FlowJo (Treestar). After gating for bacteria, median fluorescent intensity (MFI) was extracted. Log MFI was plotted against log dilution factor and four-parameter logistic curves were fitted using Prism 9/10 (GraphPad). From these curve fits, specific titers were calculated as the dilution factor giving an above-background signal (typically MFI = 1000).

Flow cytometry analysis of the K1 capsule

Bacterial overnight cultures were diluted 1:100 in fresh LB and grown at 37°C with 180 rpm shaking for 2 hours. Cells were pelleted and washed once with PBS/0.02% azide/1% BSA. 10^5 cells were added to a 96-well plate with conical bottoms (TPP) and incubated with monoclonal anti-polysialic acid antibody (ENZ-ABS560, Enzo, 1:1000 dilution) for 1 hour on ice. Cells were washed 3 times with PBS/0.02% azide/1% BSA and thereafter incubated for 1 hour on ice with the Alexa-Fluor647 polyclonal rabbit anti-mouse IgG (AB_2340241, Jackson Immuno, 1:100 dilution). Cells were washed 3 times with PBS and resuspended in 100 µl PBS. Flow cytometry acquisition was performed using a Beckman Coulter Cytoflex S with SSC and FSC parameters in logarithmic scale. Gates used to calculate "Percent anti-PSA-stained cells" were set using Ec^{K1}Δ*kps* as negative control.

Analysis of fecal Lipocalin-2 levels by ELISA

Lipocalin-2 (LCN-2) levels in feces were detected using the Mouse Lipocalin-2/NGAL DuoSet kit (R&D Systems). Feces were homogenized as described above and centrifuged 5 minutes at 17'000x *g*. LCN-2 levels were analyzed in undiluted and 1:5 and 1:10 dilutions. 96-well ELISA plates (ThermoFisher) were

coated with 100 µl capture antibody (4 µg/ml) and incubated at 4°C overnight. The plates were washed 3 times with PBS/0.05% Tween20 and blocked with 300 µl PBS/1%BSA for 3 hours at RT. The plates were washed 3 times with PBS/0.05% Tween20 and 100 µl the dilutions of the feces and the LCN-2 standard was added and incubated overnight at 4°C. The next day, the plates were washed 6 times with PBS/0.05% Tween20 before adding 100 µl of the detection antibody (500 ng/ml). The plates were incubated for 1 hour at RT, washed 6 times with PBS/0.05% Tween20 and 100 µl Streptavidin-HRP was added. After incubation for 1 hour at RT protected from sunlight, the plates were washed 6 times with PBS/0.05% Tween20. 100 µl of 1:1 mix of TMB and H_2O_2 was added, and the plates were incubated until desired signal was reached (20 minutes) at RT before adding 50 µl 1M H_2SO_4 to stop the reaction. The optical density at 450 nm was measured in Tecan microplate reader.

Statistical analysis

All mouse experiments were carried out with at least two independent repetitions, using at least two mice per group per experiment. Unless otherwise specified, researchers were not blinded for the assignment of the experiments or analysis. All statistical analyses were performed using Prism 9/10 (GraphPad) or R 4.5.2. All data was tested for normal distribution or log-normal distribution using Shapiro-Wilk normality test. If the data was log-normally distributed, statistical analysis was performed on log normalized data. For comparison of two groups of data, Mann-Whitney tests or paired t-tests were used. Kruskal-Wallis tests followed by Dunn's correction for multiple testing were used for comparisons of three or more groups. For analysis of time-course data, mixed effect analysis with Bonferroni corrections for multiple corrections was performed in Prism. If the time-course data was not normally distributed, a generalized linear mixed-effect model was applied in R using the glmmTMB library[107], assuming "Gamma" or "tweedie" distribution with log-link and post-hoc pair-wise comparisons between groups each day using the emmeans library[108]. Choice of "family" and "link" was confirmed by analyzing the distribution of the residuals using the DHARMa library[109] with Kolmogorov–Smirnov (KS), dispersion and outlier tests to evaluate deviations of observed residuals from expected residuals.

Data availability

The genomes of the phages are publicly available on Genbank with accession numbers: $\Phi_{100\text{-}A}$; PQ476009, $\Phi_{NW\text{-}B1}$; PQ476010, $\Phi_{NW\text{-}E1}$; PQ476011. All raw data generated in this study are available in the Source Data file also accessible on the open access repository Zenodo (https://zenodo.org/records/18244367). Sequencing data are accessible on the NCBI server under BioProject number PRJNA1402325.

Code availability

The R script for statistical analysis is available on the open access repository Zenodo (https://zenodo.org/records/18244367).

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Competing interest

The authors declare no competing interests.

Figure legends

Figure 1. K1-specific phages drive evolution of a capsule-less population with increased susceptibility to serum killing and attenuated virulence.

a. Growth of *E. coli* K1 O75:H5 Sequence type 1193 (Ec^{K1}) in presence of Φ_{100}, $\Phi_{NW\text{-}E1}$, $\Phi_{NW\text{-}B1}$ or the $K1\Phi_{cocktail}$ at multiplicity of infection (M.O.I.) = 0.01 compared to growth without phage measured over 18 hours (*n* = 3 independent biological replicates). Line at mean, shaded areas show standard deviation. b. Colony morphology of encapsulated and capsule-less *E. coli* K1 after plating on salt-free LB agar medium. As indicated, capsule-less mutants exhibit a translucent colony morphology visible to the naked eye. c. Plaque assay (performed in 24-well plate) used to assess phage susceptibility of clones forming colonies with opaque (wild-type) and translucent (capsule-less) morphology, demonstrating that translucent clones were resistant to phage infection d. Percent encapsulated Ec^{K1} after 18 hours (*n* = 3 independent biological replicates) of growth in presence and absence of phages, quantified by counting colonies with translucent morphology, showing fixed capsule mutants after phage exposure. e. Survival of Ec^{K1}, $Ec^{K1}\Delta kps$ and an isolated capsule-less clone with mutations in *neuC* in human serum compared to heat-inactivated serum, demonstrating increased susceptibility to serum killing when the capsule is lost. Two-tailed paired *t*-tests: $*p < 0.05$, ns = not significant, *n* = 3 independent biological replicates, dotted line at detection limit, error bars show standard deviation. f. Survival curves of mice after subcutaneous injection of 10^8 CFU of Ec^{K1}, $Ec^{K1}\Delta kps$ or an evolved Ec^{K1} with a mutation in *neuC* (Ec^{K1} *neuC**, 4 independent experiments, *n* = 35 per group), line at probability of survival, shade and dotted lines represent 95% confidence intervals, $p < 0.0001$. Capsule-less mutants are less virulent than wild-type *E. coli* during sepsis.

OD: optical density, Φ: phage, h: hour, CFU: colony forming unit, D.L.: detection limit, p.i.: post infection.

Figure 2. Capsule-specific phages drive virulence attenuation through capsule loss in the gut of colonized mice.

a. Experimental set-up of oral infection of antibiotic-pretreated LCM mice with Ec^{K1} and subsequent oral phage treatment with the $K1\Phi_{cocktail}$ (red diamonds, *n* = 8) compared to no phage control (black circles, *n* = 8) with fecal sampling for 8 days post infection. Pooled data from 4 independent experiments. b. Percent capsule-less mutants in the fecal Ec^{K1} population, determined by translucent colony morphology, demonstrating loss of capsule after oral phage treatment. c. Fecal bacterial loads of Ec^{K1}, showing no significant difference between control and phage treated groups. Statistical analysis for b and c was performed with two-tailed mixed-effect analysis of untransformed (b) and log-transformed (c) data with Bonferroni correction for multiple testing: ns = not significant, $*p < 0.05$, $***p = 0.0007$, $****p < 0.0001$. d. Fecal phage loads determined by plaque assay. If no phage is detected, the value of 10^3 PFU/g_{feces} was plotted. Box plots of the interquartile range with whiskers to min and max, line at median. Dotted line at detection limit, shaded area represents range of detection limits.

ABX: Oral antibiotics treatment, Φ: phage, p.i.: post infection, CFU: colony forming units, PFU: plaque forming units, D.L.: detection limit.

Figure 3. Exclusion of Ec^{K1} by EcN in mice vaccinated against capsule-less Ec^{K1} and treated with capsule-specific phages.

a. Overview of the experimental design. Mice were vaccinated with peracetic acid-killed Ec^{K1}Δ*kps* (PA-Ec^{K1}Δ*kps*) or mock-vaccinated with PBS weekly for 4 weeks, followed by oral infection with EcN, Ec^{K1} and the $K1\Phi_{cocktail}$ or exhausted LB. Naïve (black circles, $n = 7$), vaccinated (pink squares, $n = 10$), naïve phage treated (blue triangles, $n = 9$), vaccinated phage treated (red diamonds, $n = 16$) mice. b. Ec^{K1}Δ*kps*-specific intestinal IgA titers determined by flow cytometry, showing significantly higher IgA titers in the vaccinated groups compared to naïve mice. Kruskal-Wallis test with Dunn's correction for multiple comparison: ns = not significant, $*p = 0.0120$, $**p = 0.0025$, $***p = 0.0004$, $****p < 0.0001$. c. Fecal bacterial load of Ec^{K1} determined by selective plating, demonstrating a reduction of Ec^{K1} in vaccinated and phage treated mice compared to all control groups. If no Ec^{K1} could be detected, the value of 10^4 CFU/g_{feces} was plotted. d. Ratio of Ec^{K1} to EcN in feces determined by selective plating, reflecting significantly lower Ec^{K1} than EcN in the vaccinated and phage treated group compared to all other group. Statistical analysis for c and d was performed with generalized linear mixed-effect model on log-normalized values with Tukey corrections for multiple comparisons ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, ns = not significant, 4 independent experiments), all p-values are listed in Source Data file. Box plots of the interquartile range with whiskers to min and max, line at median.

DF: dilution factor, MFI: median fluorescent intensity, ABX: oral antibiotics treatment, D.L.: detection limit, CFU: colony forming unit, p.i.: post infection, Φ: phage.

Figure 4. Competitive exclusion, enhanced by vaccination and capsule-specific phages, of Ec^{K1} from the mother's gut microbiota protects pups from colonization after birth.

a. Experimental set-up. Females were orally vaccinated with PA-Ec^{K1}Δ*kps* or mock-vaccinated with PBS weekly for 4 weeks. Mating occurred after 3 vaccine doses. After vaccination, the females were colonized with EcN, Ec^{K1} and received the $K1\Phi_{cocktail}$ or exhausted LB, with additional doses on day 3, 6 and 7 post infection. Fecal bacterial loads were tracked for 7 days post infection. Part of the litter was euthanized on day of life (D.O.L.) 10. The fecal bacterial load in the remaining pups was tracked until D.O.L. 21 by rectal swabbing and overnight enrichment in LB. Naïve (black circles, $n = 16$), vaccinated (pink squares, $n = 11$), naïve phage treated (blue triangles, $n = 10$), vaccinated phage treated (red diamonds, $n = 21$) mice. b. Fecal loads of Ec^{K1} and (c) fecal ratio of Ec^{K1} to EcN in pregnant females, determined by selective plating ($n = 6$ per group). d. Intestinal loads of Ec^{K1} in pups at D.O.L. 10 from naïve ($n = 13$), naïve phage treated ($n = 19$), vaccinated ($n = 22$) and vaccinated phage treated ($n = 13$) mothers. When no Ec^{K1} was detected, the value of 10^4 CFU/g_{feces} is plotted. Kruskal-Wallis test with Dunn's correction for multiple testing: ns = not significant, $**p < 0.01$, $***p < 0.001$. Dotted line at detection limit, grey area represents range of detection limits. e. Fraction of pups colonized with Ec^{K1} at D.O.L. 11 to D.O.L. 21. Statistical analysis for c and d was performed with generalized linear mixed-effect model on log-normalized values with Tukey corrections for multiple comparisons: ns

= not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, 3 independent experiments. Box plots of the interquartile range with whiskers to min and max, line at median. All p-values are listed in Source Data file.

PA: peracetic acid-killed, CFU: colony forming unit, p.i.: post infection, Φ: phage, D.L: detection limit, D.O.L.: day of life.

Table 1. Primers used in this study

Identifier	Sequence 5' --> 3'	Purpose	Reference
OMD21_755	ATGTCTGAAAGACATTTACCTGATGACCAGAGCAGTACTAtgtaggctggagctgcttcg	Deletion of capsule cluster by λ *red* recombineering	This work
OMD21_756	TTGGTAGCTGTTAAGCCAAGGGCGGTAGCGTACCTGAAGAatgggaattagccatggtcc		This work
OMD21_761	ATAAGCATGGACTGACCATGG	Capsule cluster deletion verification	This work
OMD21_762	CCTGCGGAAATAATTTCTGCTG		This work
OMD21_782	GCATGTGGAAGAGGTGATTG	Insertion of apramycin at the glms site verification	This work
OMD21_783	AATGTCTCCTGGGAGGATTC		This work
OMD23_100	agttcggagacttttgacaatgctaagagaaactccagaaCATATGAATATCCTCCTTAG	Deletion of *neuC* by λ *red* recombineering	This work
OMD23_101	tggtacattccgggatgtcataaaaatctttgtaatctttGTGTAGGCTGGAGCTGCTTC		This work
OMD22_082	ATACGTAACTGGATCTAGAGC	Verification of *neuC* deletion	This work
OMD22_083	TCATATGAATCAACATCTATCTGC		This work
OMD23_059	ttactatatcggctgaaattaatgaggtcatacccaaatgCATATGAATATCCTCCTTAG	Deletion of *emrR* by λ *red* recombineering	This work
OMD23_060	tcttttataaatctggatttttgagcgagatgacgcgttaGTGTAGGCTGGAGCTGCTTC		This work
OMD23_057	gtgatggcgattatataaccc	Verification of *emrR* deletion	This work
OMD23_058	tcatattgttctccacgatc		This work

Table 2. Plasmids used in this study

Published plasmids used in this study	Description	Reference
pCP20	FLP recombinase expression plasmid, temperature sensitive ori101 repA101ts replicon; AmpR, CmR	92
pKD3	frt-cat-frt template plasmid, oriR6Kγ replicon; CmR	92
pKD4	frt-aphT-frt template plasmid, oriR6Kγ replicon; KmR	92
pDK46	I-SceI-aph-frt template plasmid, oriR6Kγ replicon; KmR	92
pBAD24	Cloning expression vector, used for AmpR; AmpR	93
pXG10-SF	pSC101 backbone carrying *PLtet0-1-gfp*; CmR	94
pGRG36	Used for site specific insertion of genes in *glms*	95
Plasmids constructed in this study		
pKD46_aprR	pKD46 with an additional apramycin casette	
pM965_kanR	Constitutive GFP expression with an additional kanamycin casette	

Editorial Summary –

E. coli K1 inhabits the human gut and can cause neonatal sepsis and meningitis. This study shows that combining oral vaccination with phages and *E. coli* Nissle excludes *E. coli* K1 from the gut and prevents its transmission from mother to neonate.

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