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# Evaluation de l'efficacité de l'éradication d'Entérobactéries productrices de BLSE du tractus digestif murin et humain par phagothérapie

François Javaudin

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NANTES UNIVERSITE

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Par

**François JAVAUDIN**

## **Évaluation de l'efficacité de l'éradication d'entérobactéries productrices de BLSE du tractus digestif murin et humain par phagothérapie**

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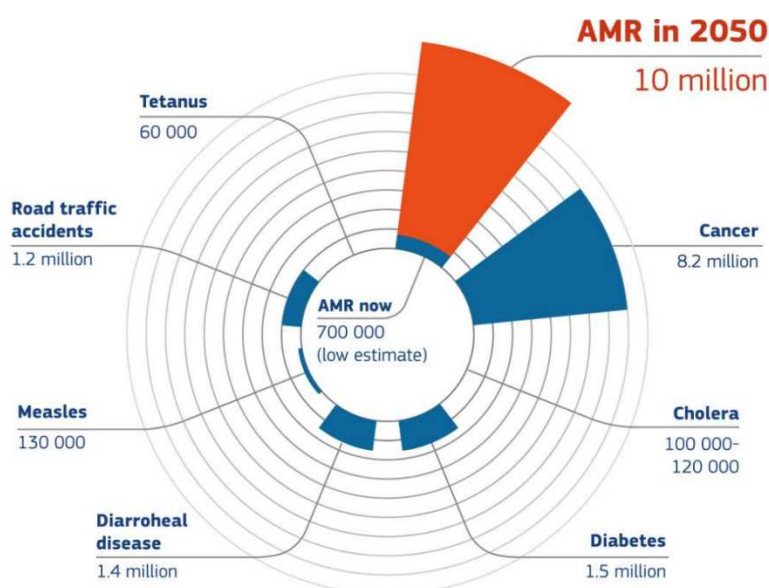
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# Introduction

## 1. Résistances bactériennes

### 1.1. Un enjeu majeur de santé publique

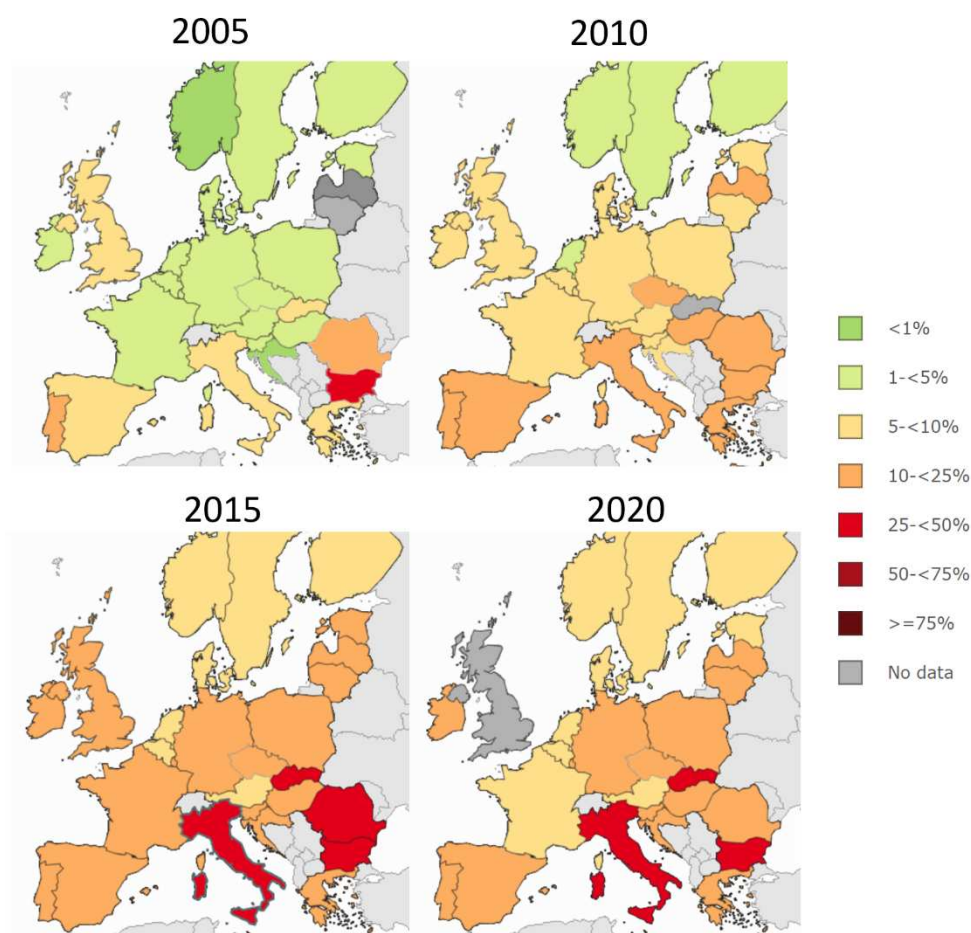
Les résistances bactériennes sont considérées comme un enjeu majeur mondial de santé publique. En décembre 2014, Jim O'Neill écrivait dans un rapport pour le gouvernement britannique que si aucune réponse globale n'était entreprise, le nombre de décès annuel attribuable à la résistance serait d'environ 10 millions par an en 2050 ; la mortalité due aux cancers serait quant à elle inférieure et estimée à 8,2 millions par an (Figure 1).(1)



**Figure 1.** Décès attribuables à l'antibiorésistance chaque année par rapport aux autres grandes causes de décès (d'après O'Neill 2014 (1)).

AMR : antimicrobial resistance

Même si ces prédictions sont critiquées,(2) Cassini *et al.* estimaient qu'en 2015 la mortalité attribuable à la résistance était en médiane de 27 249 cas contre 11 144 cas en 2017 (augmentation d'un facteur 2,46) avec en tête de liste les infections à *Escherichia coli* (*E. coli*) résistant aux céphalosporines de troisième génération (C3G). L'Italie et la Grèce étaient les deux pays européens les plus touchés (Figure 2).(3) En France la densité d'incidence des entérobactéries productrices de bêta-lactamases à spectre étendu (EBLSE) pour 1000 jours d'hospitalisations est passée de 0,13 en 2002 à 0,71 en 2016.(4)

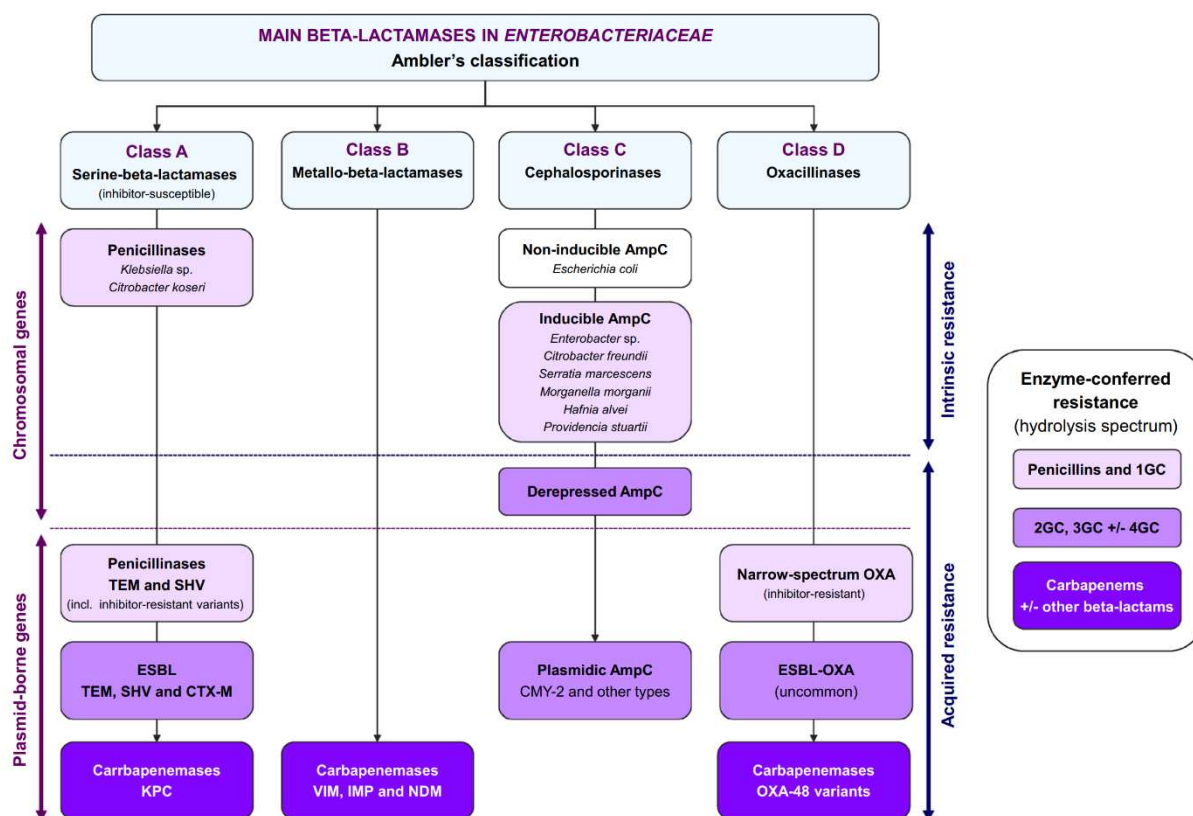


**Figure 2.** Evolution du taux d'*E. coli* résistants aux céphalosporines de 3<sup>ème</sup> génération en Europe de 2005 à 2020 (d'après l'European Centre for Disease Prevention and Control, <https://www.ecdc.europa.eu>).

## 1.2. Bêtalactamases à spectre étendu (BLSE)

Il n'existe pas de consensus sur la définition précise des BLSE. Il est couramment entendu que les BLSE sont des  $\beta$ -lactamases capables de conférer une résistance aux pénicillines, aux céphalosporines et à l'aztréonam (mais pas aux céphamycines ni aux carbapénèmes).(5) En revanche l'activité hydrolytique des BLSE peut être inhibée par l'acide clavulanique et le tazobactam.

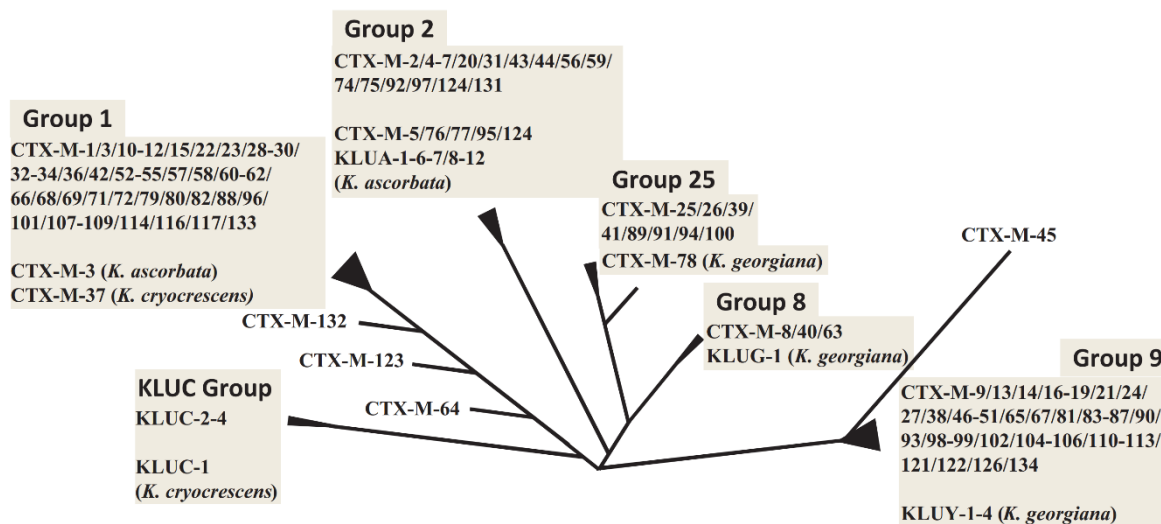
Il existe plusieurs classifications des  $\beta$ -lactamases dont deux qui sont usuellement retenues : la classification d'Ambler (structurale, basée sur des caractéristiques moléculaires ; figure 3) et la classification de Bush-Jacoby-Medeiros (fonctionnelle, basée sur des critères phénotypiques).(6,7)



**Figure 3.** Principales  $\beta$ -lactamases chez les entérobactéries d'après la classification d'Ambler (d'après Ruppé *et al.* 2015 (8)).

Les BLSE font partie de la classe A de la classification d'Amblar mais on retrouve aussi les enzymes de type OXA qui appartiennent à la classe D. Parmi les BLSE de la classe A on retrouve une homologie moléculaire variant de 20 à plus de 99% ce qui en fait un ensemble hétérogène.(9)

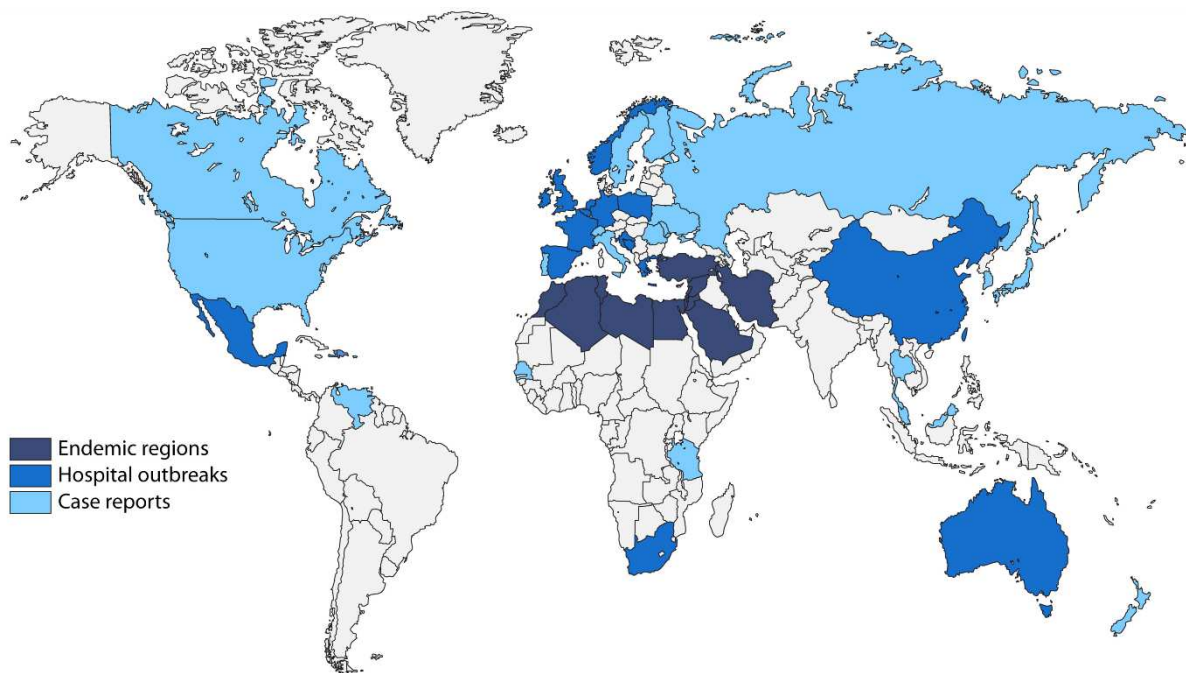
Ce sont principalement chez les entérobactéries que les BLSE ont été isolées, toutefois quelques descriptions sur d'autres bactéries Gram négatives ont été reportées (*Pseudomonas aeruginosa* par exemple).(10) Les premières bêta-lactamases ont été découvertes en Grèce dans les années 1960 avec la mise en évidence d'un plasmide à partir d'hémocultures d'un patient nommé Temoniera d'où l'appellation TEM. Depuis les années 1990 un autre type de BLSE nommé CTX-M (CefoTaXime-Munich) a fait son émergence et est devenu l'enzyme de résistance BLSE prédominante notamment chez *E. coli*. (11,12) La famille des CTX-M comprend six groupes principaux (n°1, 2, 25, 8, 9 et KLUC) (Figure 3). Parmi ces groupes de CTX-M, l'enzyme CTX-M-15 est la plus fréquemment isolée au niveau mondial.



**Figure 3.** Schéma montrant la similarité entre les enzymes de la lignée CTX-M et le regroupement en différents groupes (d'après D'Andrea *et al.* 2013 (13)).

### 1.3. La résistance OXA-48

La carbapénémase OXA-48 a été initialement décrite chez *Klebsiella pneumoniae* en 2003,(14) puis chez *Escherichia coli*.(15) A cette époque elle a été à l'origine de multiples foyers épidémiques dans les hôpitaux d'Istanbul et d'Ankara.(14–16) En 2010 les premières épidémies françaises ont été rapportées dans la littérature.(17) Il s'agit d'une carbapénémase de classe D (Figure 2) dont un plasmide est la source principale du gène *bla*<sub>OXA-48</sub>.(18) Elle a une activité modérée contre les carbapénèmes et n'hydrolyse pas les C3G. Cependant OXA-48 est fréquemment associée à d'autres bêta-lactamases comme des BLSE.(15,17) En France, depuis 2009, la part du mécanisme OXA-48 est en augmentation et est désormais le plus fréquent chez les entérobactéries productrices de carbapénémases (78% entre 2004 et 2015).(19) En Europe, les dernières données de 2019, retrouvaient un taux de résistance aux carbapénèmes de *K. pneumoniae* très hétérogène selon les pays : estimé à 58,3% en Grèce (pays le plus touché) et à 1,0% en France ; pour *E. coli* ces taux étaient plus bas avec 1,6% en Espagne et à 0,04% en France.(20) Mondialement, les zones endémiques d'entérobactéries OXA-48 sont matérialisées par l'Afrique du Nord et le Moyen Orient (Figure 4).



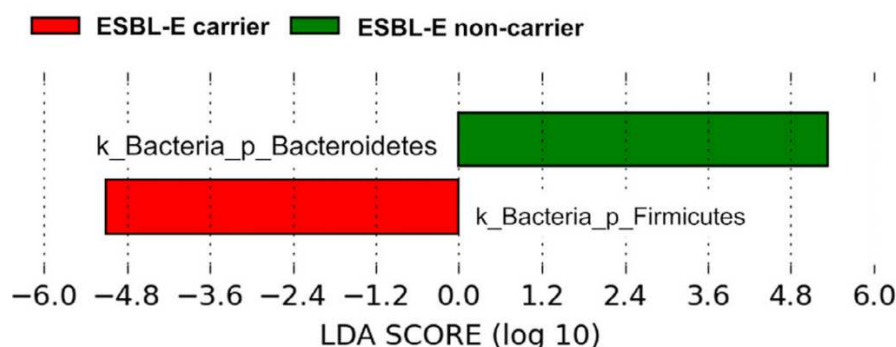
**Figure 4.** Distribution mondiale des entérobactéries avec OXA-48 (d'après Pitout *et al.* 2019 (21)).

## 2. Microbiote digestif et résistome

Le microbiote intestinal est composé de milliers d'espèces bactériennes, dont certaines sont cultivables. Cependant, la majorité ne l'est pas et nécessite d'autres techniques d'analyse.(22) Le microbiote intestinal est reconnu pour être un réservoir ainsi qu'un lieu propice aux transferts de gènes de résistances aux antibiotiques, d'où le concept de résistome.(23) Le taux de colonisation digestive en entérobactéries BLSE chez les sujets sains varie considérablement en fonction des pays ; il est de 2% en Amérique contre 46% en Asie. Les facteurs associés au risque de colonisation sont essentiellement : l'exposition à une antibiothérapie dans les 4 à 12 mois précédents ainsi que les voyages internationaux.(24) Les perturbations du microbiote, communément appelées dysbiose, peuvent favoriser la colonisation ou l'infection du tractus digestif.(25,26) Une étude menée en



Thaïlande comparait la composition du microbiote intestinal (analyse du métagénome par séquençage de l'ARN 16S) et retrouvait des différences entre les personnes colonisées ou non par entérobactéries BLSE. Les sujets colonisés présentaient une diminution significative en *Bacteroidetes* et une augmentation des *Firmicutes* (Figure 5).(27)



**Figure 5.** Différences d'abondance relative en *Bacteroidetes* et *Firmicutes* entre les sujets colonisés ou non par entérobactéries BLSE (d'après Piewngam *et al.* 2019 (27)).

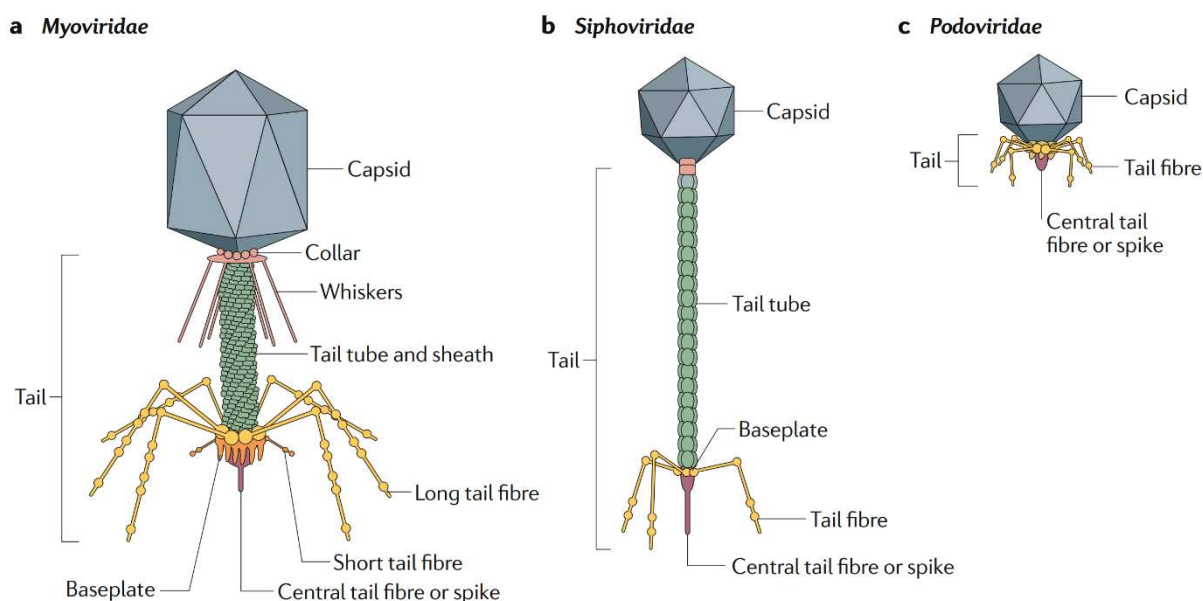
Actuellement l'European Society of Clinical Microbiology and Infectious Diseases (ESCMID) et l'European Committee on Infection Control (EUCIC) ne recommandent pas de pratiquer en routine des stratégies de décolonisation digestive des patients porteurs de bactéries Gram négatives multirésistantes.(28) En effet, les différentes stratégies de décolonisation étudiées se basent surtout sur une antibiothérapie (colistine, tobramycine et gentamycine notamment) et pourraient par conséquent être associées à l'émergence de résistance.(29,30) De plus, l'effet de la décolonisation digestive semble modéré et instable dans le temps.(31) L'utilisation de probiotiques ou la réalisation de transplantations fécales sont également des stratégies évoquées ; mais le manque de preuves solides n'a pas pu intégrer une de ces stratégies en pratique courante. En effet, les experts suggèrent une évaluation plus poussée de leur efficacité à plus long terme ainsi que de leurs potentiels effets

indésirables. Ils évoquent aussi le développement urgent de nouvelles stratégies de décontamination digestive dont la phagothérapie.(28)

### 3. Phagothérapie

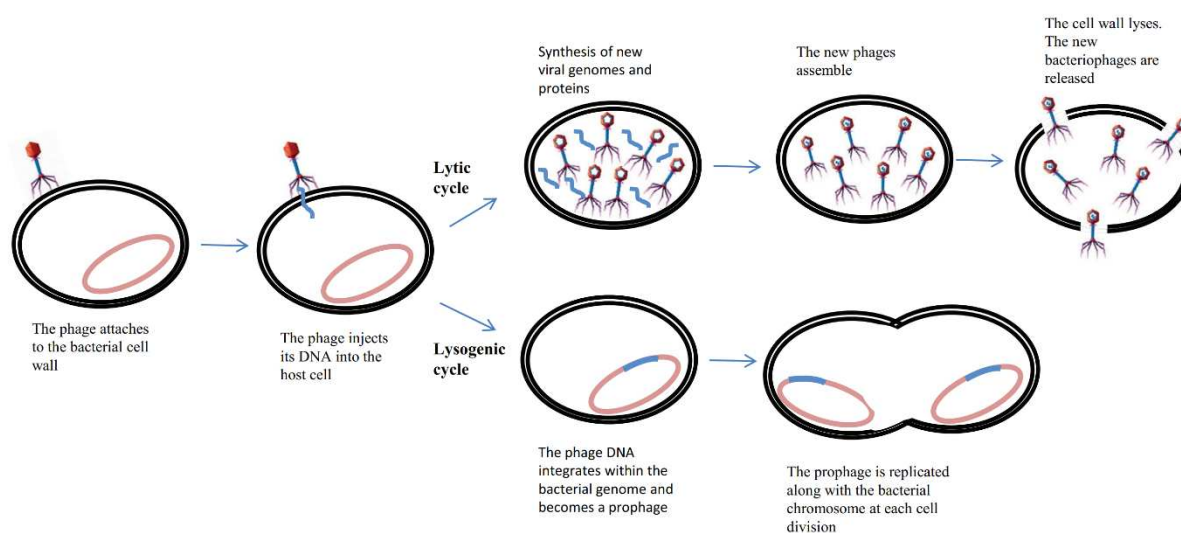
#### 3.1. Généralités

Les phages, ou bactériophages, sont des virus antibactériens utilisés depuis 1917 par Félix d'Hérelle à l'institut Pasteur dans le traitement des bacilles dysentériques.(32) Ils s'attaquent d'une manière spécifique à une ou certaines bactéries sans être capable d'infecter les cellules eucaryotes. Ce sont principalement des virus à ADN et plus rarement à ARN. La majorité des phages décrits ont une morphologie comprenant une tête et une queue avec un génome à ADN double brin (Figure 6). Ils appartiennent à l'ordre des *Caudovirales* qui comprend 14 familles et un genre d'après la classification de l'International Committee on Taxonomy of Viruses (ICTV) de 2021. (33,34)



**Figure 6.** Morphologie des 3 principaux groupes des phages à queue (d'après Nobrega *et al.* 2018 (35))

L'acide nucléique viral pénètre dans le cytoplasme de la bactérie cible après l'adsorption du phage sur celle-ci. Le cycle lytique comprend ensuite la phase de synthèse, ou d'éclipse, avec initialement transcription d'ARNm précoces codant pour des protéines précoces nécessaires à la synthèse de l'ADN du phage et l'hydrolyse de l'ADN bactérien. Vient ensuite la phase de maturation avec assemblage des particules phages, puis la phase de lyse et de libération avec destruction de la bactérie et libération des phages produits (Figure 7).(36,37) Les phages tempérés sont eux à l'origine d'un cycle lysogénique où le génome du phage s'intègre à celui de son hôte et reste dormant en tant que prophage (Figure 6). Ce génome viral se réplique avec son hôte et peut occasionnellement, dans des conditions spécifiques, entrer dans un cycle lytique.(38) La lysogénie et les prophages peuvent être bénéfiques pour la bactérie en encodant par exemple des gènes de résistance aux antibiotiques ou bien encore des facteurs de virulence.(39)



**Figure 7.** Représentation des cycles lytique et lysogénique (d'après Kakasis *et al.* 2019 (38)).

L'utilisation des phages par voie orale semble sûre avec un impact minimal sur le microbiote intestinal.(40) Ils peuvent également être utilisés par voie intraveineuse sans que d'effets indésirables graves n'aient été décrits.(41)

Actuellement les bactériophages sont toujours utilisés empiriquement et historiquement en médecine humaine dans les pays d'Europe de l'Est, notamment en Géorgie, alors qu'ils ont été abandonnés dans le monde occidental au profit de l'antibiothérapie.(32)

### 3.2. Infection et décolonisation digestive

Une seule étude humaine a évalué l'intérêt d'une phagothérapie par voie orale dans le traitement des diarrhées bactériennes à *E. coli* entéropathogènes mais l'efficacité du traitement n'était pas supérieure au placebo ni d'un point de vue clinique ni d'un point de vue bactériologique.(42) Devant les premiers résultats négatifs l'étude a été stoppée prématurément après l'inclusion et l'analyse de 120 sujets (225 inclusions étaient initialement prévues). Les auteurs ont conclu que les connaissances sur l'interaction in-vivo entre phages et bactéries étaient actuellement insuffisantes pour espérer maîtriser une telle thérapeutique.(42)

Un cas clinique a été publié en 2020 où un patient de 57 ans a été décolonisé avec succès grâce à une phagothérapie orale et rectale contre *K. pneumoniae* multirésistant (KPC-3).(43) Cette stratégie suscite des espoirs thérapeutiques mais dont les preuves en médecine humaine sont actuellement inexistantes.(44) En revanche de nombreux modèles animaux ont été décrits notamment au sein d'élevages de volailles (environ 50 publications en 2021) en ciblant principalement les infections à *Salmonella enterica* et *Campylobacter jejuni*.(45) La phagothérapie, dans ce contexte, semblaient efficaces sur le plan microbiologique en diminuant

significativement la concentration en bactéries en moyenne de  $0,82 \log_{10}$  CFU/g au niveau du cæcum.(45) Chez les mammifères de nombreux modèles ont également été développés (environ 40 publications en 2021). Nous les avons analysés dans une revue de la littérature en y incluant des modèles infectieux et de colonisation digestive.(46) Nous nous sommes concentrés sur les mammifères car le microbiome intestinal diffère entre les mammifères et les oiseaux.(47) Notre analyse de l'impact microbiologique des phages s'est essentiellement focalisée sur les échantillons les plus distaux du tractus digestif, c'est-à-dire la concentration bactérienne présente dans les selles de préférence. En effet, cela résume non seulement l'effet global sur l'ensemble de l'appareil digestif mais aussi évalue la dissémination bactérienne dans l'environnement.

### 3.3. Revue de la littérature : « Intestinal Bacteriophage Therapy: Looking for Optimal Efficacy »

Ci-dessous l'article publié dans Clinical Microbiology Reviews (CMR, IF 2020 = 26.132) en octobre 2021.(46)



# Intestinal Bacteriophage Therapy: Looking for Optimal Efficacy

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**SUMMARY** Several human intestinal microbiota studies suggest that bacteriophages, viruses infecting bacteria, play a role in gut homeostasis. Currently, bacteriophages are considered a tool to precisely engineer the intestinal microbiota, but they have also attracted considerable attention as a possible solution to fight against bacterial pathogens resistant to antibiotics. These two applications necessitate bacteriophages to reach and kill their bacterial target within the gut environment. Unfortunately, exploitable clinical data in this field are scarce. Here, we review the administration of bacteriophages to target intestinal bacteria in mammalian experimental models. While bacteriophage amplification in the gut was often confirmed, we found that in most studies, it had no significant impact on the load of the targeted bacteria. In particular, we observed that the outcome of bacteriophage treatments is linked to the behavior of the target bacteria toward each animal model. Treatment efficacy ranges from poor in asymptomatic intestinal carriage to high in intestinal disease. This broad range of efficacy underlines the difficulties to reach a consensus on the impact of bacteriophages in the gut and calls for deeper investigations of key parameters that influence the success of such interventions before launching clinical trials.

**KEYWORDS** enteric pathogens, gastrointestinal infection, intestinal colonization

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## INTRODUCTION

Advances in sequencing technologies have revealed the diversity of microbes in human-associated microbiota, in particular in the gut (1–4). Among these microbes, bacteria and their viruses, bacteriophages (phages), are the most abundant. Recently, several studies have shown an association between variations of intestinal phage communities and several human diseases or disorders such as inflammatory bowel disease (IBD), colorectal cancer, or child growth impairment (5–12). Although causality between intestinal phages and human diseases has not been established, the most convincing data supporting an active role for phages come from the field of fecal microbiota transplantation, used to treat *Clostridioides difficile* recurrent infections. Indeed, sterile fecal filtrate was found to be as effective as nonfiltered fecal transplantation, and phages from the donors could colonize the recipient's microbiota for at least 12 months (13, 14). More unexpected was the recent evidence that a prophage sequence from the intestinal bacterium *Enterococcus hirae* triggers a specific immune response, enhancing the cyclophosphamide treatment of cancer patients, showing that the role of phages in the gut is broader than anticipated (15).

Interestingly enough, the link between gut and phages roots from the original 1917 report of F. d'Herelle, who isolated novel ultramicrobes from feces of patients recovering from diarrhea caused by *Shigella* and named them bacteriophages (16). Following this report, phage therapy, the use of phages to treat bacterial infections, increased for a few decades before antibiotics became the most successful antibacterial treatment. Unfortunately, the growing expansion of bacteria becoming resistant to antibiotics is now recognized as a major public health threat for which solutions must be found. During the last 2 decades, this situation has given more weight to phage therapy, which is now becoming more accessible in Europe and the United States to patients in lack of other solutions (17–19). For more than 80 years, phage therapy treatments have also been used in Georgia and few other countries in Eastern Europe that can provide access to such treatment. However, no recent and well-documented clinical data are available impeding the translation of this clinical experience worldwide.

Given the initial observation by d'Herelle, one could expect that phage therapy of intestinal bacterial infections would be the most documented treatment. However, this is not the case. Worse, a unique recent (<30 years old) clinical trial that attempted to treat *Escherichia coli* diarrhea in Bangladeshi children failed (20). This failure could not be attributed to the lack of efficacy of phages targeting *E. coli*. Indeed, the authors uncovered an unexpected correlation between the abundance of strains of *Streptococcus* and diarrhea symptoms, questioning the direct causality of *E. coli* strains in this disease (21, 22). This observation considerably reduced the number of children in which an effect on the phage treatment could be found (20). In addition, the clinical status of children who had *E. coli* as the main pathogen was not dramatically improved upon treatment. Although this clinical trial and associated safety studies have firmly established that oral application of phages is safe (23), we must recognize a gap of knowledge in translating the *in vitro* efficacy of phages into *in vivo* optimal conditions, in particular when targeting intestinal bacterial pathogens.

Here, we focused this review on the literature reporting intestinal phage therapy experiments using exclusively mammalian gut models, since such models are generally considered as proxies to study the physiopathology of intestinal diseases in humans. Only a limited number of studies showed unambiguously that a phage treatment led to a significant reduction of the targeted bacteria coupled with amplification of phages and reduction of symptoms, which are the criteria fulfilling the definition of an active phage therapy treatment (24, 25). Given the anatomy of the digestive tract and the spatial distribution of microbes (75), it seems unlikely that passive phage therapy, which relies on the administration of single high dose of phages to reduce the bacterial load within a single infection cycle, would indeed be sufficient to provide an effective treatment in this organ. The different modalities that have been tested were also reviewed to delineate recommendations for future studies. Overall, we found that the success of phage treatments was more frequent in infected animals developing a disease than in animals merely or strongly colonized by an intestinal pathogen and not developing clinical signs of disease. We then aligned this analysis with the



**TABLE 1** Experimental phage treatments of *C. difficile*-infected animals

Animals	Bacterial challenge and phage therapy <sup>a</sup>	<i>C. difficile</i> (CFU g <sup>-1</sup> ) and outcomes	Reference
Hamsters	Bacterial challenge: oral 10 <sup>3</sup> CFU and a second after 2 wks Group a: 1 phage, oral 10 <sup>8</sup> PFU at 0 h Group b: 1 phage, oral 10 <sup>8</sup> PFU at 0 h and every 8 h up to 48 h Group c: 1 phage, oral 10 <sup>8</sup> PFU at 0 h and every 8 h up to 72 h	Control: 4 × 10 <sup>5</sup> ; all dead ≤ 72 h a: 7 × 10 <sup>4</sup> ; no death b: 7 × 10 <sup>5</sup> ; no death c: 4 × 10 <sup>5</sup> ; 1 dead ≤ 72 h All animals died after subsequent bacterial challenge	32
Hamsters	Bacterial challenge: oral 2 × 10 <sup>3</sup> CFU Group a: 2 to 4 phages, oral 10 <sup>8</sup> PFU ml <sup>-1</sup> at 0 h and every 8 h up to 36 h	Control: 10 <sup>6</sup> –10 <sup>7</sup> ; death ≤ 55 h a: 10 <sup>4</sup> –10 <sup>5</sup> ; death ≤ 88 h	33
Mice	Bacterial challenge: oral 10 <sup>5</sup> spores Group a: oral 1 wild-type phage at 4 h and twice daily during 4 days Group b: oral 1 recombinant phage at 4 h and twice daily during 4 days Group c: oral 1 mutant wtPhage at 4 h and twice daily during 4 days Group d: oral 1 mutant crPhage at 4 h and twice daily during 4 days	Control: 10 <sup>8</sup> a: 10 <sup>8</sup> b: 10 <sup>8</sup> (transient 1-log reduction) c: 10 <sup>7</sup> d: 10 <sup>7</sup>	34

<sup>a</sup>The bacterial challenge was initiated at  $t = 0$ .

growing literature that identified some of the parameters affecting the efficacy of phages in the gut. We concluded that using phages as a treatment to cure intestinal infections or as tools for precisely engineering the intestinal microbiota remains a challenging concept that requires deeper mechanistic investigations.

### SYNTHESIS OF REPORTS ON THE EFFICACY OF PHAGE TREATMENTS TARGETING INTESTINAL PATHOGENS IN MAMMALS

The state of the art of molecular mechanisms of phage infection relevant to phage therapy has been abundantly reviewed over the past few years (17, 27–29). However, these mechanisms were rarely studied within the context of the mammalian gut ecosystem. We reviewed the literature of experimental phage therapy studies on bacterial pathogens causing intestinal infections in mammals in order to highlight conditions of treatments and point to mechanisms involved in phage therapy efficacy.

#### *Clostridioides difficile*

*C. difficile* is an intestinal pathogen for which phage therapy would be particularly useful given its growing resistance to antibiotics (30). Since no virulent (strictly lytic) phage of *C. difficile* has been isolated thus far, studies were performed using temperate phages (Table 1), which are usually disregarded for therapy because of their ability to transfer undesirable bacterial genes such as those coding toxins or antibiotic resistance systems (31).

Two studies were performed with hamsters and reported a longer survival rate upon phage treatment. Ramesh et al. showed that 17 of the 18 phage-treated animals survived the bacterial challenge for 2 weeks, while all untreated animals died within 72 h. The first oral administration of phages (10<sup>8</sup> PFU) was performed immediately after the bacterial challenge, but in the absence of a longitudinal record of phages in feces along the experiment it remains unclear whether phage amplified. In addition, at the endpoint of the experiment, the *C. difficile* levels in ceca were similar in all groups (about 10<sup>5</sup> CFU per ml [CFU ml<sup>-1</sup>]), and phages were detected in only few animals, including untreated controls. Unsurprisingly, phage-treated animals were not protected by a second bacterial challenge administered 2 weeks after phage treatment (32). Using a four-phage cocktail (8 × 10<sup>7</sup> PFU), Nale et al. reported a decrease of *C. difficile* counts in the lumens of the ceca and colons of phage-treated animals compared to control hamsters 36 h after the first phage dose (an ~2-log CFU ml<sup>-1</sup> reduction). In agreement with these data, phage-treated animals survived up to 120 h compared to 60 h for the untreated controls (33). In a third and more recent study, a temperate phage was genetically modified to behave as a virulent phage, and its oral application in mice 2 days postchallenge led to a significant reduction of ~2 logs CFU g<sup>-1</sup> of *C. difficile* levels in feces compared to controls and the wild-type phage-treated group (34).

The two studies performed with hamsters used similar protocols, but the authors observed opposite outcomes. While phage treatments were roughly comparable in



**TABLE 2** Experimental phage treatments of *E. faecalis*-infected animals

Animals	Bacterial challenge and phage therapy <sup>a</sup>	<i>E. faecalis</i> (CFU g <sup>-1</sup> )	Reference
Mice	Group a: 1 phage, oral 1 × 10 <sup>10</sup> PFU by oral gavage after a 6 h colonization period and 5 × 10 <sup>8</sup> PFU ml <sup>-1</sup> in drinking water	Control: 8 × 10 <sup>9</sup> a: 7 × 10 <sup>9</sup> (transient 3-fold reduction)	35

<sup>a</sup>The bacterial challenge was initiated at  $t = 0$ .

dose and frequency, the nature of the *C. difficile* inoculum was different: Nale et al. used spores, while Ramesh et al. used cells (32, 33). When phages were administered shortly after the challenge bacteria, a rapid decrease of the infectious dose was expected since bacteria would not have the time to adapt to the gut environment. However, spores, which are resistant to phages, may challenge this expectation, and this may explain why the study by Nale et al. resulted only in a time shift to death. Finally, a recent study performed in mice with genetically modified temperate phages demonstrated that a strong reduction of their ability to form lysogens resulted in a higher efficacy at reducing the levels of *C. difficile* in the gut compared to the wild type (34).

### ***Enterococcus faecalis***

Duerkop et al. observed a modest but significant reduction,  $\sim 0.7$ -log CFU g<sup>-1</sup>, of the fecal levels of *E. faecalis* in mice 24 h after administration of a single phage once (10<sup>10</sup> PFU) 6 h after bacterial gavage of germfree mouse drinking water supplemented with 10<sup>5</sup> PFU ml<sup>-1</sup> of the same phage (35) (Table 2). However, from 48 h to 9 days post-treatment, *E. faecalis* fecal levels were no longer different between phage-treated and control groups. These authors also noticed that some *E. faecalis* clones became phage resistant as soon as 48 h posttreatment and that high levels of phages persisted in the feces ( $> 10^6$  PFU g<sup>-1</sup>).

### ***Escherichia coli***

Within the broad genetic diversity of the *E. coli* species that includes both pathogenic and commensal strains (36), several studies using different animal models focused on the highly pathogenic strains of the serotype O157:H7, which were analyzed together, while strains from other serotypes included mostly murine models and were also analyzed together (Table 3).

***Escherichia coli* O157:H7. (i) Mice (three studies).** Tanji et al. (37) observed in phage-treated groups (single or repeated administrations started 2 days postchallenge) a decrease in *E. coli* fecal concentration of  $\sim 1$  log CFU g<sup>-1</sup> 5 days after bacterial challenge that was not observed 6 days later. Dissanayake et al. (38) obtained similar results within 1 day with an administration of phages twice a day before and after the bacterial challenge. In both studies, the impact of phage treatment on fecal level of *E. coli* was less pronounced at later time points (11 and 3 days, respectively). On the other hand, Sheng et al. (39) reported a prolonged impact on fecal *E. coli* levels of a single or two phages treatment started 24 h after the bacterial challenge and administered once a day during few days. Indeed, *E. coli* clones were detected in phage-treated groups up to 10 days only if an enrichment step was performed, while the untreated group excreted over 10<sup>2</sup> CFU g<sup>-1</sup>.

**(ii) Sheep (five studies).** Three studies showed no significant difference between phage-treated and control groups (39–41). Sheng et al. administered a single phage four times, starting 1 day after bacterial challenge and followed the animals for 21 days, after which phages were still detectable in the feces (39). Bach et al. reported the administration of a single dose of a single phage 2 days after the bacterial challenge, and monitored the animals for 28 days after phage treatment (40). The concentration of phages rapidly decreased and reached the detection threshold within 2 days. Raya et al. also used a single dose of a single phage, but they administered this dose 3 days after the bacterial challenge with a 2-day follow-up after phage treatment (41). In another study, Raya et al. reported a significant reduction in *E. coli* levels of 3 log CFU g<sup>-1</sup> throughout the gut 2 days after phage administration by a single dose on animals challenged 5 days before. Concomitantly, 10<sup>3</sup> to 10<sup>6</sup> PFU ml<sup>-1</sup> of phages were detected in the feces (42). Callaway et al. administered a single dose of an eight-phage cocktail at three different doses at 48 h and at 72 h postchallenge

**TABLE 3** Experimental phage treatments of *E. coli* O157:H7-infected animals

Animals	Bacterial challenge and phage therapy <sup>a</sup>	<i>E. coli</i> O157:H7 (CFU g <sup>-1</sup> )	Reference
Sheep	Bacterial challenge: oral 10 <sup>8</sup> CFU Group a: 1 phage, oral 10 <sup>13</sup> PFU at day 2	Control: undetectable a: undetectable	40
Mice	Bacterial challenge: oral 10 <sup>9</sup> CFU Group a: 3 phages, oral 10 <sup>8</sup> PFU at day 2 Group b: 3 phages, oral 10 <sup>10</sup> PFU at day 2 Group c: 3 phages, oral 10 <sup>10</sup> PFU at day 2 and then daily	Control: 10 <sup>2</sup> –10 <sup>3</sup> a: 10 <sup>2</sup> –10 <sup>3</sup> (transient 1-log reduction) b: 10 <sup>2</sup> –10 <sup>3</sup> (transient 1-log reduction) c: 10 <sup>1</sup> –10 <sup>2</sup>	37
Group a: sheep	Bacterial challenge a and c: oral 3.5 × 10 <sup>10</sup> CFU; b: oral 10 <sup>8</sup> CFU; c: rectal 10 <sup>10</sup> CFU a: 1 phage, oral 10 <sup>11</sup> PFU at days 1, 9, 10, and 11	Control for group a: undetectable a: undetectable	39
Group b: mice	b: 1 phage, oral 10 <sup>10</sup> PFU at days 1, 2, and 3	Control for groups b and c: <10 <sup>1</sup> b: undetectable (transient 3-log reduction)	
Group c: mice	c: 2 phages, oral 10 <sup>10</sup> PFU at days 1, 2, and 3	c: undetectable (transient 2-log reduction)	
Group d: steers	d: 2 phages, rectal 10 <sup>11</sup> PFU at days 8, 9, 10, and 15+ in drinking water 10 <sup>6</sup> PFU ml <sup>-1</sup> from day 8	Control d: 10 <sup>2</sup> d: 10 <sup>2</sup> (transient 1-log reduction)	
Sheep	Bacterial challenge: oral 10 <sup>10</sup> CFU Group a: 1 phage, oral 10 <sup>11</sup> PFU at day 3	Control: 10 <sup>6</sup> a: 10 <sup>4</sup>	41
Sheep	Bacterial challenge: oral 10 <sup>10</sup> CFU Group a: 8 phages, oral 10 <sup>9</sup> PFU at 48 h and 72 h  Group b: 8 phages, oral 1:1 PFU/CFU at 48 h and 72 h  Group c: 8 phages, oral 10:1 PFU/CFU at 48 h and 72 h Group d: 8 phages, oral 100:1 PFU/CFU at 48 h and 72 h	Control for group a: 10 <sup>5</sup> a: 10 <sup>4</sup> Control for groups b, c, and d: 10 <sup>3</sup> b: 10 <sup>1</sup> c: 10 <sup>2</sup> d: 10 <sup>3</sup>	43
Steers	Bacterial challenge: oral 5 × 10 <sup>10</sup> CFU Group a: 4 phages, oral 3.3 × 10 <sup>11</sup> PFU at days -2, 0, 2, 6, and 9 Group b: 4 phages, rectal 1.5 × 10 <sup>11</sup> PFU at days -2, 0, 2, 6, and 9 Group c: 4 phages, oral and rectal 4.8 × 10 <sup>11</sup> PFU at days -2, 0, 2, 6, and 9	Control: <10 <sup>1</sup> a: <10 <sup>1</sup> b: <10 <sup>1</sup> c: <10 <sup>1</sup>	44
Steers	Bacterial challenge: oral 10 <sup>11</sup> CFU Group a: 4 encapsulated phages, oral 10 <sup>10</sup> PFU at days -1, 1, 3, 6, and 8 Group b: 4 encapsulated phages, in feed 10 <sup>10</sup> PFU at days -1, 1, 3, 6, and 8	Control: <10 <sup>1</sup> a: <10 <sup>1</sup> b: <10 <sup>1</sup>	46
Steers	Bacterial challenge: oral 10 <sup>10</sup> CFU Group a: 2 phages, oral 10 <sup>11</sup> PFU at days 1, 2, and 3	Control: 10 <sup>1</sup> Group a: 10 <sup>1</sup>	45
Sheep	Bacterial challenge: oral 10 <sup>10</sup> CFU Group a: 2 phages, oral 10 <sup>11</sup> PFU, at day 3 Group b: 1 phage naturally present in the gut	Control: 10 <sup>6</sup> a: 5 × 10 <sup>3</sup> b: 2 × 10 <sup>3</sup>	42
Mice	Bacterial challenge: oral 10 <sup>8</sup> CFU Group a: 16 phages, oral 10 <sup>9</sup> PFU at -2.5 h, 2.5 h and twice daily during 3 days Group b: cocktail of 16 phages, 10 <sup>8</sup> PFU at -2.5 h, 2.5 h and twice daily during 3 days Group c: cocktail of 4 phages, 10 <sup>9</sup> PFU at -2.5 h, 2.5 h, and twice daily during 3 days	Control: undetectable a: undetectable (transient 0.2-log reduction) b: undetectable (transient 0.2-log reduction) c: undetectable (transient 0.2-log reduction)	38

<sup>a</sup>Bacterial challenge was initiated at  $t = 0$ .

and found that the *E. coli* levels were significantly reduced at 72 h postchallenge (i.e., 24 h after the first phage administration) but not at later time points. However, at 96 h postchallenge, the bacterial loads in cecal and rectal contents were ~2 log g<sup>-1</sup> lower in the phage-treated group given the lowest phage dose but not in the group given the 100-fold-higher dose (43).

**(iii) Steers (four studies).** Three studies reported no difference between oral phage-treated and control groups over periods from 7 to 30 days posttreatment (44–46). The fourth study reported an initial decrease in fecal *E. coli* levels between 1 and 10 days after oral and rectal phage treatment, which did not last during the next 3 weeks (39). In all reports, phages isolated from feces were still able to infect the O157:H7 *E. coli* strain, but their impact on the *E. coli* intestinal load progressively decreased over time, independently of the administration route and frequency.

**Other *Escherichia coli* serotypes. (i) Mice/rats (nine studies).** Only three studies reported a significant reduction from 1 to 2 log g<sup>-1</sup> in the *E. coli* loads in feces from phage-treated animals by a single oral administration compared to controls (47–49). These data were independent of the administration time after bacterial challenge, being 1, 3, 8, or 10 days (Table 4). Two studies were performed with a cocktail of three phages (47, 48), while the third study used a single phage (49). In all studies, both phages and the targeted *E. coli* were still detected in feces, up to 2 weeks after phage treatment for the Galtier study (48).

The six other studies showed no (50–53) or only transient (54, 55) reductions in the fecal levels of *E. coli*. This reduction was dose dependent in the study of Maura et al. since a 100-fold reduction in the oral phage dose had no significant impact 24 h after phage administration compared to controls (54). Nevertheless, fecal levels of phages remained roughly stable over 2 weeks at ~10<sup>7</sup> PFU g<sup>-1</sup> (52). By administering a cocktail of 140 phages continuously in drinking water, or three times a day for 20 days after bacterial challenge, Abdulmir et al. observed a transient reduction of *E. coli* fecal titers of ~2 log CFU ml<sup>-1</sup> between days 5 and 8, followed by a progressive increase until day 20. Parallel to this bacterial increase, the fecal titer of phages decreased from day 8 and was no longer detectable on day 20 (55). In all other studies (50, 51, 53), whether the phage was administered alone or in a cocktail or before or after the bacterial challenge, no significant reduction in *E. coli* fecal levels was observed or only in some parts of the gut, such as the stomach or the small intestine.

**(ii) Other species (rabbit, pig, calf, and lamb—five studies).** Zhao et al. (56) noticed a 15-fold decrease in the cecal levels of challenged bacteria 3 days after administration of a single oral dose of one phage to rabbits or a single oral dose of 20 mg of ciprofloxacin. Using pigs, which developed diarrhea upon administration of an *E. coli* strain, Jamalludeen et al. (57) reported a significant decrease in the *E. coli* fecal level 6 days postchallenge compared to the control when a single dose of one phage was orally administered, but not with a three-phage cocktail. Surprisingly, the fecal levels of phages following the single phage treatment were below the threshold of detection, whereas they were 1 to 2 log g<sup>-1</sup> above the threshold of detection after the treatment with the cocktail. Nevertheless, the authors reported a reduction in the diarrhea score with both treatments. With three animal species and a different *E. coli* serotype for each, Smith et al. (58) observed a reduction in animal mortality congruent with a lower bacterial shedding when they administered one or two phages once the animals showed signs of diarrhea.

**Synthesis of experiments with *E. coli* (all serotypes).** In mice, seven studies reported a decrease in *E. coli* levels in phage-treated groups, whereas four studies showed no effect, and one study did not include enough animals to be conclusive (53). In most cases, the levels of the *E. coli* strain introduced in the guts of mice remained low and sometimes tended to decrease within a few days. Under these conditions, the impact of phage application on *E. coli* levels was often significant (37–39, 47–49). In contrast, studies performed with models of high and stable levels of *E. coli* gut colonization showed weak or no phage impact (51–54). Therefore, the data showed that the density of the target bacteria critically affects the efficacy of phages in the murine gut.

Experiments performed with sheep revealed the same shortcoming of unstable *E. coli* gut colonization seen in mice and lasting no more than 1 week. Interestingly, despite no significant impact on *E. coli* fecal titers, oral phage administration reduced the *E. coli* levels in intestinal organs (41–43). With steers, *E. coli* colonization was more stable over several days, with a decline observed only after several weeks. Nevertheless, weak or no efficacy of phages was also observed (44–46). In studies with pigs and calves, *E. coli* gut colonization was stable, and phages administered orally led to a significant reduction in *E. coli* titers in the feces (57, 58). In

**TABLE 4** Experimental phage treatments of *E. coli*-infected animals

Animals	Bacterial challenge and phage therapy <sup>a</sup>	<i>E. coli</i> (CFU g <sup>-1</sup> )	Reference
Group a: calves	Bacterial challenge for a: oral $3 \times 10^9$ CFU; b and c: oral $3 \times 10^8$ CFU a: 2 phages, oral $10^{11}$ PFU at 1 h, at 8 h, or at onset of diarrhea	Control for group a: $10^{10}$ ; mortality 95% a: $4 \times 10^4$ ; mortality 35%	58
Group b: piglets	b: 2 phages, oral $10^{10}$ PFU at the onset of diarrhea	Control for group b: $5 \times 10^7$ ; mortality 60% b: $10^5$ ; mortality 0%	
Group c: lambs	c: one phage, oral $10^9$ to $10^{10}$ PFU at 8 h	Control for group c: $4 \times 10^9$ ; mortality 30% c: $6 \times 10^7$ ; mortality 0%	
Mice	Bacterial challenge for a, b, c, and d: oral $5 \times 10^7$ CFU; e and f: oral $10^4$ CFU Group a: 4 phages, in drinking water $10^6$ PFU ml <sup>-1</sup> Group b: 4 phages, in drinking water $10^6$ PFU ml <sup>-1</sup> + ampicillin $20 \mu\text{g ml}^{-1}$ during the first week Group c: 4 phages, in drinking water $10^6$ PFU ml <sup>-1</sup> + ampicillin starting at day -3 Group d: 1 phage, in drinking water $10^5$ PFU ml <sup>-1</sup> , at day 7 Group e: 1 phage, in drinking water $10^5$ PFU ml <sup>-1</sup> , at day 0 Group f: 1 phage, in drinking water $10^5$ PFU ml <sup>-1</sup> , at day -7	Control for group a: undetectable a: undetectable Control for group b: $10^3$ to $10^5$ b: undetectable Control for group c: undetectable to $10^7$ c: undetectable to $10^1$ d: $10^8$ to $10^4$ after treatment (no control group) e: $10^9$ and $10^6$ after treatment (no control group) f: $10^9$ (no control group)	53
Mice	Bacterial challenge: oral $10^{10}$ CFU Group a: cocktail of 3 phages, $10^9$ PFU ml <sup>-1</sup> in drinking water	Control: $10^5$ to $10^8$ a: $10^5$ to $10^8$	50
Mice	Bacterial challenge: oral $5 \times 10^7$ CFU Group a: 2 phages, oral $5 \times 10^4$ PFU after colonization Group b: 2 phages, oral $10^7$ PFU after colonization Group c: 1 phage, oral $10^6$ PFU after colonization Group d: 1 phage, oral $10^8$ PFU after colonization Group e: 1 phage, oral $10^6$ PFU at day -7 Group f: 1 phage, oral $10^6$ PFU at day 3	Control for group a: $10^6$ a: $10^6$ b: $3 \times 10^9$ (data not shown for control group) c: same as control (data not shown) Control for group d: $10^7$ d: $10^7$ e: $10^9$ (no control group) f: high titers (data not shown)	51
Pigs	Bacterial challenge: oral $10^{10}$ CFU Group a: 1 phage, oral $10^{10}$ PFU at 15 min Group b: 3 phages, oral $10^9$ PFU at 15 min Group c: 2 phages, oral $10^8$ PFU at 24 h, 30 h, and 36 h	Percentage of <i>E. coli</i> excretion Control for group a: 83% a: 3 to 37% Control for group b: 63% b: 38% Control for group c: 21% ( $10^8$ CFU g <sup>-1</sup> ) c: 80% ( $10^7$ CFU g <sup>-1</sup> )	57
Mice	Bacterial challenge: oral $10^6$ CFU Group a: 3 phages, in drinking water $3 \times 10^8$ PFU ml <sup>-1</sup> at day 3 during 24 h	Control: $10^8$ a: $10^8$	52
Mice	Bacterial challenge: oral $10^6$ CFU Group a: 3 phages, in drinking water $3 \times 10^8$ PFU ml <sup>-1</sup> at day 3 during 24 h Group b: 3 phages, in drinking water $3 \times 10^{10}$ PFU ml <sup>-1</sup> at day 3 during 24 h	Control: $10^9$ a: $10^9$ b: $10^9$ (transient 3-fold reduction)	54
Rats	Bacterial challenge: in drinking water $10^8$ CFU ml <sup>-1</sup> Group a: 140 phages, in drinking water $10^7$ PFU ml <sup>-1</sup> during 20 days Group b: 140 phages, oral $4 \times 10^7$ PFU, three times per day during 20 days Group c: 140 phages, oral capsules $5 \times 10^6$ PFU, three times per day during 20 days	Control: $3 \times 10^5$ a: $3 \times 10^5$ (transient 2-log reduction) b: $3 \times 10^5$ (transient 2-log reduction) c: $5 \times 10^5$ (transient 4-log reduction)	55
Mice	Bacterial challenge: oral $10^7$ CFU Group a: 1 phage, oral $2 \times 10^7$ PFU at day 4	Control for groups a and b: $10^9$ a: $10^9$	47

(Continued on next page)

**TABLE 4** (Continued)

Animals	Bacterial challenge and phage therapy <sup>a</sup>	<i>E. coli</i> (CFU g <sup>-1</sup> )	Reference
	Group b: 3 phages, oral 2 × 10 <sup>7</sup> PFU at day 4 Group c: 3 phages, oral 6 × 10 <sup>5</sup> PFU at day 7	b: 10 <sup>8</sup> Control for groups c and d: 10 <sup>8</sup> c: 10 <sup>5</sup> d: 10 <sup>5</sup>	
	Group d: 3 phages, oral 6 × 10 <sup>7</sup> PFU at day 7		
Mice	Bacterial challenge for a: oral 10 <sup>9</sup> CFU; b and c: oral 10 <sup>8</sup> CFU Group a: 3 phages, oral 3 × 10 <sup>7</sup> PFU at day 1 (2 doses) Group b: 3 phages, oral 3 × 10 <sup>7</sup> PFU at day 8 Group c: 3 phages, oral 3 × 10 <sup>7</sup> PFU at day 10	Control: 10 <sup>8</sup> a: 10 <sup>6</sup> b: 10 <sup>6</sup> c: 10 <sup>5</sup>	48
Rabbits	Bacterial challenge: oral 10 <sup>10</sup> CFU Group a: 1 phage, oral 10 <sup>10</sup> PFU, at day 3	<i>eaeA</i> gene concn (10 <sup>-6</sup> μg/μl) Control: 16.0 a: 1.1	56
Mice	Bacterial challenge: oral 4 × 10 <sup>6</sup> CFU Group a: 1 phage, oral 4 × 10 <sup>8</sup> PFU, at 24 h	Control: 8 × 10 <sup>8</sup> a: 5 × 10 <sup>7</sup>	49

<sup>a</sup>The bacterial challenge was initiated at  $t = 0$ .

the latter two animals, *E. coli* intestinal colonization was associated with a disease; this was not the case for any of the sheep, steer, or mouse models. This observation suggests that the pronounced efficacy of phage treatments under such conditions could be linked to the additive action of the immune defense against the pathogen. An alternative explanation would be that the physiology of the pathogen causing the disease may favor phage replication.

### *Listeria monocytogenes*

A single study with mice reported that a moderate dose (10<sup>5</sup> PFU) of a six-phage cocktail administered daily before and after the bacterial challenge led to a decrease in the fecal level of *L. monocytogenes* 3 days after bacterial challenge (Table 5). This decrease was as strong as the one observed for antibiotic-treated animals. However, phage titers in feces and cecal contents were below the threshold of detection. This is consistent with a weak amplification of phages caused by the low abundance of *L. monocytogenes* in intestinal organs, i.e., ~10<sup>2</sup> CFU g<sup>-1</sup> (59).

### *Pseudomonas aeruginosa*

In a murine model of intestinal sepsis, a single phage was administered orally once (Table 6). The observations at 10 days postchallenge revealed that a pretreatment 1 day before or after the challenge did not affect the survival rate of animals compared to untreated controls. In contrast, a treatment administered 6 days postchallenge increased the survival up to 66%. The fecal concentrations of *P. aeruginosa* were only significantly reduced by <1 log CFU g<sup>-1</sup> for both postchallenge treatments (60).

### *Salmonella enterica* Serovar Typhimurium

All of the studies included ( $n = 7$ ) were performed with pigs with the goal to propose phages for lowering food contamination (Table 7). Three studies reported a significant reduction of *S. Typhimurium* levels in gut sections (ileum and cecum) but not in the feces of phage-treated animals compared to control groups at both 6 and 48 h posttreatment (61–63). In these studies, phages were administered by either oral, intramuscular, or intraperitoneal routes a few hours after the bacterial challenge. Saez et al. used microencapsulated phages orally administered three times every 2 h postchallenge and observed a significant reduction in *Salmonella* counts of 2 logs in the ileum at 6 h postchallenge compared to untreated animals.

**TABLE 5** Experimental phage treatments of *L. monocytogenes*-infected animals

Animals	Bacterial challenge and phage therapy <sup>a</sup>	<i>L. monocytogenes</i> (CFU g <sup>-1</sup> )	Reference
Mice	Bacterial challenge: oral 10 <sup>5</sup> CFU Group a: 6 phages, oral 1 × 10 <sup>5</sup> PFU daily from days -3 to 3	Control: 90 a: <10	59

<sup>a</sup>The bacterial challenge was initiated at  $t = 0$ .

**TABLE 6** Experimental phage treatments of *P. aeruginosa*-infected animals

Animals	Bacterial challenge and phage therapy <sup>a</sup>	<i>P. aeruginosa</i> (CFU g <sup>-1</sup> )	Reference
Mice	Bacterial challenge: in drinking water 10 <sup>8</sup> CFU ml <sup>-1</sup> during 3 days Group a: 1 phage, oral 10 <sup>10</sup> PFU at day -1 Group b: 1 phage, oral 10 <sup>10</sup> PFU at day 1 Group c: 1 phage, oral 10 <sup>10</sup> PFU at day 6	Control: 3 × 10 <sup>5</sup> a: 2 × 10 <sup>5</sup> b: 2 × 10 <sup>4</sup> c: 5 × 10 <sup>4</sup>	60

<sup>a</sup>The bacterial challenge was initiated at  $t = 0$ .

When phages were administered daily for 5 days before the bacterial challenge, their levels 6 h postchallenge reached higher values in ileal and cecal contents compared to animals that received phages every 2 h postchallenge ( $>10^6$  PFU ml<sup>-1</sup> versus  $>10^3$  PFU ml<sup>-1</sup>) (63). The study performed by Callaway et al. reported a trend toward a reduction in *Salmonella* counts at 96 h postchallenge, which was, however, not significant. These researchers used a cocktail of two phages administered orally 24 and 48 h after the bacterial challenge, and the phage levels reached 10<sup>4</sup> PFU ml<sup>-1</sup> in the intestinal contents at 96 h (64). Using a six-phage cocktail administered at different concentrations 2 days postchallenge, Albino et al. observed no impact of *Salmonella* counts compared to controls 18 h later (65). When phages were mixed with food ( $2 \times 10^9$  PFU kg<sup>-1</sup>), pigs displayed lower fecal shedding scores than did the control groups on days 7 and 14 postchallenge (66). At 6 h after the administration of 15 microencapsulated phages, Wall et al. reported that *Salmonella* counts in ileal and cecal samples were reduced, but all feces remained positive for *Salmonella* in phage-treated and control groups (67).

Most of the experiments reported were performed to address a highly specific challenge: the suitability of a phage application prior to meat processing (61, 62, 65, 67). Therefore, experimental settings included phage administration only 6 h before sacrifice but did not always

**TABLE 7** Experimental phage treatments of *S. Typhimurium*-infected animals

Animals	Bacterial challenge and phage therapy <sup>a</sup>	<i>S. Typhimurium</i> (CFU g <sup>-1</sup> )	Reference
Pigs	Bacterial challenge: intranasal 10 <sup>8</sup> CFU Group a: 26 phages, intraperitoneal $1.2 \times 10^9$ PFU at 18 h Group b: 26 phages, intramuscular $1.2 \times 10^9$ PFU at 18 h Group c: 26 phages, oral $1.2 \times 10^9$ PFU at 18 h Group d: 1 phage, oral and intramuscular $2 \times 10^{10}$ at 3 h	Control for groups a, b, and c: 10 <sup>4</sup> a: $5 \times 10^4$ b: $7 \times 10^3$ c: $5 \times 10^{43}$ Control for group d: $2 \times 10$ d: $<10^2$	61
Pigs	Bacterial challenge: intranasal $5 \times 10^8$ CFU Group a: 1 phage, oral and intramuscular $2 \times 10^{10}$ PFU at 3 h	Control: $2 \times 10^3$ a: $<10^2$	62
Pigs	Bacterial challenge for a: oral $5 \times 10^8$ CFU; b: oral $5 \times 10^9$ CFU Group a: 15 microencapsulated phages, oral 10 <sup>10</sup> PFU at 0 h, 2 h, and 4 h Group b: 15 microencapsulated phages, oral 10 <sup>10</sup> PFU at 48 h, 50 h, and 52 h	Control for group a: $4 \times 10^3$ a: 3 Control for group b: $8 \times 10^2$ b: $3 \times 10^1$	67
Pigs	Bacterial challenge: oral 10 <sup>9</sup> CFU Group a: 1 phage, in feed $2 \times 10^9$ PFU kg <sup>-1</sup>	Shedding score (0 to 3) Control: 1.4 a: 0.2	66
Pigs	Bacterial challenge: oral $5 \times 10^9$ CFU Group a: 14 microencapsulated phages, in feed $5 \times 10^{11}$ PFU per day from day -5 to day 0 Group b: 14 microencapsulated phages, oral $5 \times 10^{11}$ PFU at 0, 2, 4, and 6 h	Control: $5 \times 10^3$ a: $5 \times 10^2$ b: $2 \times 10^3$	63
Pigs	Bacterial challenge: oral $2 \times 10^{10}$ CFU Group a: 2 phages, oral $3 \times 10^9$ PFU at 24 h and 48 h	Control: $2 \times 10^1$ a: 2	64
Pigs	Bacterial challenge: oral $5 \times 10^5$ CFU (2 doses) Group a: 6 phages, orally (a) 10 <sup>3</sup> PFU ml <sup>-1</sup> at day 3 Group b: 6 phages, orally (a) 10 <sup>5</sup> PFU ml <sup>-1</sup> at day 3 Group c: 6 phages, orally (a) 10 <sup>7</sup> PFU ml <sup>-1</sup> at day 3 Group d: 6 phages, orally (a) 10 <sup>9</sup> PFU ml <sup>-1</sup> at day 3	Control: $3 \times 10^3$ a: $3 \times 10^3$ b: $3 \times 10^3$ c: $3 \times 10^3$ d: $3 \times 10^3$	65

<sup>a</sup>The bacterial challenge was initiated at  $t = 0$ .

**TABLE 8** Experimental phage treatments of *S. sonnei*-infected animals

Animals	Bacterial challenge and phage therapy <sup>a</sup>	<i>P. aeruginosa</i> (CFU pellet <sup>-1</sup> )	Reference
Mice	Bacterial challenge: oral 10 <sup>8</sup> CFU Group a: 5 phages, oral 10 <sup>9</sup> PFU at -1 h Group b: 5 phages, oral 10 <sup>9</sup> PFU at 1 h Group c: 5 phages, oral 10 <sup>9</sup> PFU at 3 h Group d: 5 phages, oral 10 <sup>9</sup> PFU at -1 h and 1 h	Control: 1114 a: lower than control (data not shown) b: 110 c: lower than control (data not shown) d: 26 (most effective group)	68

<sup>a</sup>The bacterial challenge was initiated at  $t = 0$ .

include the intestinal readout of such treatment. The results showed that *Salmonella* levels in the gut tend to decrease, but never significantly, compared to the controls. Encouragingly, the only long-term study (2 weeks), during which the authors analyzed the impact of phages in pigs carrying *Salmonella* showed a significant reduction of the *Salmonella* titers in fecal samples (66).

### *Shigella sonnei*

The oral administration to mice of a commercial product including five phages (ShigActive) 1 h before or after the bacterial challenge, or both, led to reduced fecal levels of *S. sonnei* 48 h postchallenge (Table 8). These regimens were more effective than ampicillin at both 24 and 48 h. It should also be mentioned that mice naturally eliminated *Shigella* within 72 h (68).

### *Vibrio cholerae*

Four studies with animal models of *V. cholerae* infection were analyzed (Table 9). In 1963, Dutta et al. noted that rabbits treated with a single phage 1 or 8 h postchallenge survived longer than untreated controls. These results were obtained with five different phages (69). In a more recent study, rabbits infected by the oral administration of *V. cholerae*

**TABLE 9** Experimental phage treatments of *V. cholerae*-infected animals

Animals	Bacterial challenge and phage therapy <sup>a</sup>	<i>V. cholerae</i> (CFU g <sup>-1</sup> ) and outcomes	Reference
Rabbits	Bacterial challenge: intrainestinal 10 <sup>4</sup> vibrios per 100 g of body wt Five different phages tested individually Group a: at -1 h Group b: at 8 h Group c: at 16 h Group d: after onset of diarrhea	Mortality rate (n/n) Control: 4/4 Phage R: a: 0/6; b: 2/8; c and d: 6/6 Phage 138: a: 0/4; b: 2/4; c and d: 4/4 Phage 145: a: 1/4; b: 4/4; c and d: 4/4 Phage 149: a: 1/4; b: 2/4; c and d: 4/4 Phage 163: a: 0/4; b: 0/4; c and d: 4/4	69
Mice	Bacterial challenge: oral 5 × 10 <sup>7</sup> CFU Group a: 5 phages, oral 1 × 10 <sup>7</sup> PFU at days 1, 2, and 3	Control: 10 <sup>10</sup> a: 9 × 10 <sup>3</sup>	72
Group a: mice	Bacterial challenge for a: oral 5 × 10 <sup>5</sup> CFU a <sub>1</sub> : 1 phage, oral 10 <sup>6</sup> -10 <sup>7</sup> at -3 h a <sub>2</sub> : 1 phage, oral 10 <sup>6</sup> -10 <sup>7</sup> at -3 h a <sub>3</sub> : 1 phage, oral 10 <sup>6</sup> -10 <sup>7</sup> at -3 h a <sub>4</sub> : 3 phages, oral 10 <sup>6</sup> -10 <sup>7</sup> at -3 h a <sub>5</sub> : 3 phages, oral 5 × 10 <sup>5</sup> to 9 × 10 <sup>5</sup> at -6 h a <sub>6</sub> : 3 phages, oral 5 × 10 <sup>5</sup> to 9 × 10 <sup>5</sup> at -12 h a <sub>7</sub> : 3 phages, oral 5 × 10 <sup>5</sup> to 9 × 10 <sup>5</sup> at -24 h a <sub>8</sub> : 3 phages, oral 10 <sup>8</sup> PFU at -6 h a <sub>9</sub> : 3 phages, oral 10 <sup>8</sup> PFU at -12 h a <sub>10</sub> : 3 phages, oral 10 <sup>8</sup> PFU at -24 h	Control for group a: 2 × 10 <sup>7</sup> to 9 × 10 <sup>7</sup> a <sub>1</sub> : 10 <sup>5</sup> a <sub>2</sub> : 10 <sup>2</sup> a <sub>3</sub> : 10 <sup>1</sup> a <sub>4</sub> : 10 <sup>1</sup> a <sub>5</sub> : 10 <sup>1</sup> a <sub>6</sub> : 4 × 10 <sup>4</sup> a <sub>7</sub> : 2 × 10 <sup>6</sup> a <sub>8</sub> : 10 <sup>5</sup> a <sub>9</sub> : 4 × 10 <sup>5</sup> a <sub>10</sub> : 8 × 10 <sup>5</sup>	71
Group b: rabbits	Bacterial challenge for b: oral 5 × 10 <sup>8</sup> CFU b <sub>1</sub> : 3 phages, oral 4 × 10 <sup>9</sup> to 8 × 10 <sup>9</sup> PFU at -3 h b <sub>2</sub> : 3 phages, oral 4 × 10 <sup>9</sup> to 8 × 10 <sup>9</sup> PFU at -24 h	Control for group b: 10 <sup>10</sup> b <sub>1</sub> : 10 <sup>2</sup> b <sub>2</sub> : 3 × 10 <sup>7</sup>	
Rabbits	Bacterial challenge: oral 5 × 10 <sup>8</sup> CFU Group a: 1 phage, oral 10 <sup>9</sup> PFU at -6 h Group b: 1 phage, oral 10 <sup>9</sup> PFU at 6 h	Control: 2 × 10 <sup>7</sup> a: 3 × 10 <sup>3</sup> b: 3 × 10 <sup>3</sup>	70

<sup>a</sup>The bacterial challenge was initiated at  $t = 0$ .

**TABLE 10** Experimental phage treatments of *Y. enterocolitica*-infected animals

Animals	Bacterial challenge and phage therapy <sup>a</sup>	<i>Y. enterocolitica</i> (CFU g <sup>-1</sup> )	Reference
Mice	Bacterial challenge: oral $2 \times 10^8$ CFU Group a: 1 phage, oral $10^9$ PFU ml <sup>-1</sup> at 6 h	Control: $10^4$ a: $10^2$	74

<sup>a</sup>The bacterial challenge was initiated at  $t = 0$ .

did not develop symptoms when a single phage treatment was given either before or after the challenge. Both treatment regimens led to a reduction in *V. cholerae* counts in the cecal fluids by 24 h postchallenge. High levels of phage replication, up to  $10^7$  PFU g<sup>-1</sup>, were detected in the gut when the treatment was performed postchallenge. Slightly lower numbers,  $\sim 10^5$  PFU g<sup>-1</sup>, were recovered with the prophylactic treatment (70). Using murine and rabbit models, Yen et al. tested a preventive phage treatment, which reduced the levels of *V. cholera* in the gut 24 h postchallenge. These researchers also showed that a three-phage cocktail performed better than did individual phages (71). In another study, a five-phage cocktail significantly decreased the levels of viable *V. cholerae* in the intestinal tissues at days 1 and 4 postchallenge of phage-treated mice compared to untreated controls (72).

Cholera is one of the earliest human infections for which phage therapy has been tested (73). Experimental results confirmed the efficacy of this treatment in both mice and hamsters (70–72). Impressively, the results are consistent among studies performed 65 years apart. Here, as with *E. coli* in calf and pig models, the efficacy of phage treatments was observed when administered to animals developing a disease.

### *Yersinia enterocolitica*

In a murine model, oral administration of a single dose of one phage 6 h after bacterial challenge resulted in a significant reduction of the bacterial load within 18 h (4-log CFU g<sup>-1</sup> reduction) in the cecum and colon (Table 10). This reduction persisted until 144 h postchallenge, but to a lesser extent (2 log CFU g<sup>-1</sup>). A limited increase in the phage titer was observed in the cecum and colon at 12 h postadministration, and a decrease was subsequently observed until 48 h, after which phages were no longer detected. No significant histopathologic lesions in the cecum and a lower level of proinflammatory cytokines were observed in phage-treated mice as opposed to the control untreated group (74).

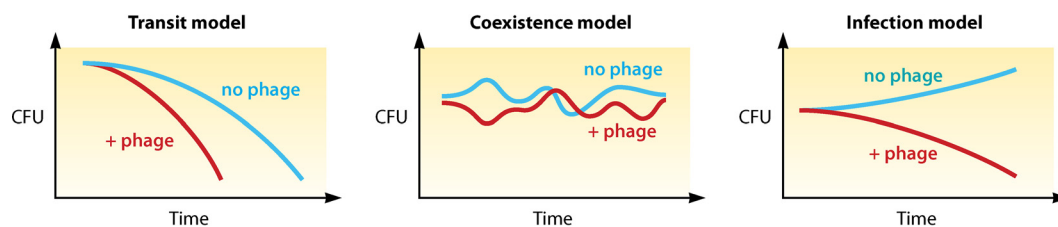
## OVERVIEW

Altogether, two-thirds of the studies discussed above showed that phage treatments had either a transient or no efficacy in reducing the levels of the target bacteria, showing clearly that intestinal phage therapy is not as easy as one could expect. Therefore, in-depth analysis of studies must be undertaken to highlight the conditions driving successful phage therapy treatments in the gut. We organized this analysis under the form of documented answers to three key questions.

### Is the Amplification of Phages in the Gut Supported by Experimental Data?

The answer to this question, which is expected to be positive given the self-amplification of phages on their target bacteria, is not so definitive. In some studies, the data are lacking, while in others, the phage levels remained relatively low, mirroring the levels of the target bacteria. Nevertheless, the transit of phages throughout the gut was not found to be a major hurdle since phages were recovered from the fecal contents of control animals. In addition, a buffer to neutralize the gastric acidity was often used (23, 51, 75). Therefore, in most of the studies phage amplification was reported. Sometimes, an increased number of phages compared to the dose administered was observed within 24 h, testifying unambiguously to phage replication (35, 47, 48, 52, 57, 70). More striking was the continuous presence of phages in feces during weeks from animals that received a single phage administration. However, the overall consequence of *in vivo* phage replication did not match with a parallel reduction in target bacteria except when animals developed intestinal diseases. In fact, our analysis of both phage and bacteria populations distinguished three situations depending on the behavior of a given bacterial strain in animals (Fig. 1): (i) when the phage-targeted





**FIG 1** Three models recapitulate the variation of the bacterial populations in the gut of mammals receiving a phage treatment (red) compared to untreated controls (blue). Following phage introduction in animals colonized by their target bacteria, three models were defined: transit, coexistence, and infection. These models correspond to the outcomes directly linked to the behavior of bacteria toward animals. Either bacteria could not stably colonize an animal's gut (transit model), or they colonized stably at high levels (coexistence model), or they induced an intestinal infection (infection model).

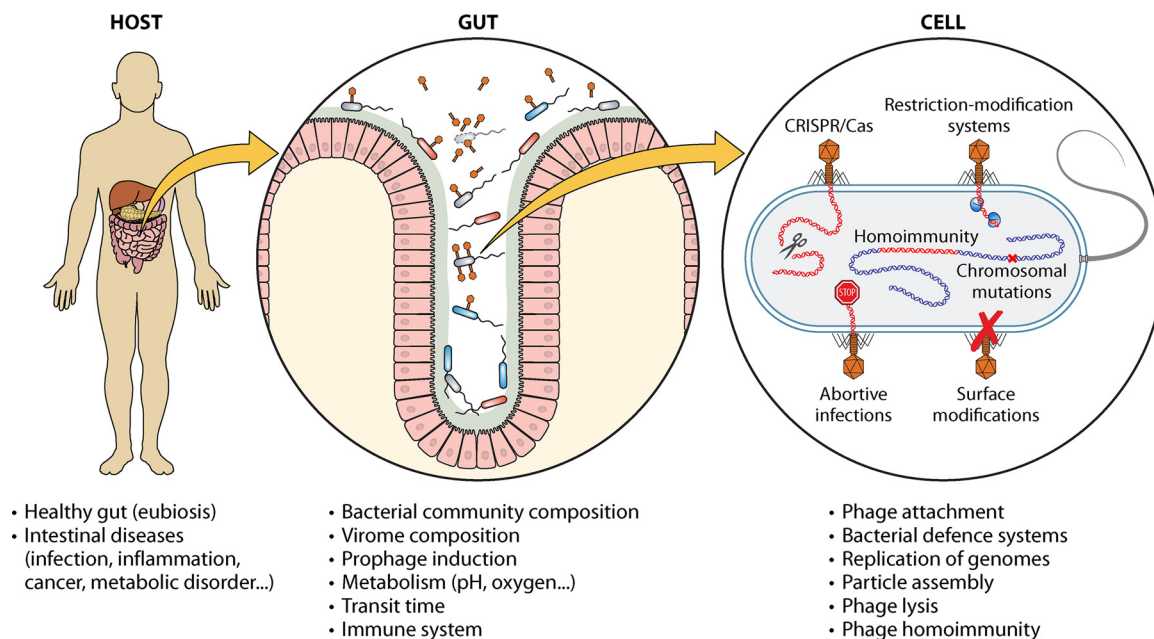
bacterium mimics the gut transit of an opportunistic pathogen with no stable colonization and without developing a disease, phage application accelerates the pace at which the bacterium is washed out (transit model); and (ii) when the bacterium stably colonizes the gut without affecting the animal's health, behaving like a commensal strain, long-term coexistence of phage and bacteria populations is observed, without major impacts on bacterial colonization levels (coexistence model). Recently, a study by Hsu et al., in which multiple bacteria were targeted altogether, reported the coexistence of phage T4 and its *E. coli* target after an initial drop of the fecal level of *E. coli*, suggesting that such coexistence is probably more frequent than anticipated (76). (iii) Finally, when intestinal bacterial infections occur, the administration of phages significantly decreases the level of pathogenic bacteria (infection model). This model would fulfill the expectations of efficient intestinal phage therapy. While in such a situation successive cycles of phage amplification may be expected, it remains possible that a single strong dose could be sufficient to reduce the burden of the targeted intestinal pathogen (passive phage therapy) and allow other antibacterials, mainly antibiotics, and the immune system to eliminate the remaining infectious bacteria.

### Are Transit, Coexistence, and Infection Models Relevant in Humans?

First, the transit model could be hard to assess during a clinical trial, since intestinal colonization may not last long enough to directly test the efficacy of phage treatments. Indeed, human intestinal viromes have revealed that phages are abundant and diverse (77). These resident phages may reduce the duration of a transient intestinal colonization or even prevent the colonization of incoming opportunistic pathogens. Nevertheless, frequent samples of feces could be collected, and interactions between intestinal phages and bacteria could be experimentally tested. A recent study of *E. coli* phages residing in the gut of children revealed that in a few cases some phages were dominating the *E. coli* phage population (78). This observation suggests that prompt amplifications of resident phages could reflect their protective role against incoming bacteria.

Second, the coexistence model is in agreement with the longitudinal study of viromes from healthy humans, revealing their long-term stability and strong link to the bacterial microbiome (79). This stability expresses the resilience of the microbiota as a community able to maintain its equilibrium state. This is a characteristic that is unique to this environment compared to others, such as skin or lungs, where the efficacy of phage therapy appears to be more promising (80, 81). Therefore, the lack of evidence from experimental models, supporting that oral phage application can efficiently decrease intestinal bacteria, may be in part related to the absence in these models of a disease context that could provide a more favorable environment for phage activity.

Third, the infection model relates to classical phage therapy treatments. Here, the goal of the phage administration is to reduce the load of the pathogen in order to restore a healthy environment. In immunocompetent animals, this would most likely rely on the synergistic action of phages and immune cells, defined as immunophage synergy, which has been experimentally demonstrated during pulmonary phage therapy but not yet during the treatment of intestinal infections (18). It is also worth mentioning that the immune response itself can provoke host damages that could be more deleterious than bacterial multiplication (82).



**FIG 2** Several factors in the host, organ, and cell influence phage activity in the gut. The health state of the host (left) imposes a global physiological environment with prolonged consequences on the intestinal microbiota including phage-bacteria interactions. In the organ (middle), the cellular environment (immune cells, other microbes) affects bacterial physiology (pH, oxygen) with direct consequences on phage dynamics (prophage induction and bacterial susceptibility to phages). Finally, in the cell (right), different bacterial defense mechanisms will impact the outcome of phage-bacterium interactions. Epithelial cells, pink; mucus layer, gray; DNA molecules correspond to bacterial (blue) and phage (red) genetic material; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR associated.

We can also hypothesize that other antibacterial defenses, such as antimicrobial peptides or commensal members of the gut microbiota (colonization resistance), would likely participate in the overall success of the treatment, as well as antibiotics, which are very often administered with phages during compassionate treatments (83).

Overall, the three proposed models sound plausible in the human context, but they await clinical data to be more firmly supported. In particular, extrapolation of data from rodent models ignores the specific behaviors of these animals in terms of food regimens and nocturnal activities, among other characteristics (84). Perhaps, more acutely, the dose of the pathogen used in experimental models is often several orders of magnitude higher than the bacterial load to which a human may be usually exposed. For instance, infecting a 20-g mouse with  $10^8$  CFU of *Salmonella* is equivalent to a human of 70 kg ingesting a piece of food contaminated by  $3.5 \times 10^{11}$  CFU, whereas the infectious dose during outbreaks in human was estimated to be in the  $10^3$ - to  $10^5$ -CFU range (85). A large inoculum in experimental models leads to an increase in the abundance of the prey population for phages but also speeds the infection of the mammalian host, which may result in lethal cellular damage despite clearance of the pathogen.

### What Are the Limiting Factors Affecting Phage Efficacy in the Gut?

The optimistic approach to rely on the amplification of phages to decrease the density of intestinal bacteria, based on their rapid *in vitro* efficacy, is not unambiguously supported by the data, suggesting that the *in vivo* efficacy of phages is jeopardized. Three main barriers to phage infection in the gut are anticipated. First, phages need to reach the gut. Second, phages must find a susceptible target. Third, phages have to achieve a successful infection. How phages will mechanistically overcome these barriers will depend not only on their nature (physical, chemical, or biological challenges) but also on the environment phages will face at three levels that are the host, the organ, and the bacterial cell (Fig. 2).

In addition to an acidic pH, which was mentioned earlier, hydrolytic enzymes can also reduce the number of phage particles transiting in the gut. In studies with a cocktail of T4-like phages fed to mice not colonized with bacteria supporting phage replication, Denou et

al. estimated that a substantial decrease in phages occurred during gut transit. These authors also observed that a 3-log-lower dose of phage administered to human volunteers still led to the detection of phages in all fecal samples, while the gastric acidity is lower in humans than in mice (50). These observations were reported multiple times with mouse models (23, 51, 75). This illustrates the necessity of performing biodistribution studies to narrow the time and numbers of phage particles reaching each gut section to get a better understanding of the phage/bacterium ratio needed for phage amplification. An example of a study that includes a large set of treatment modalities was published by Smith et al. in 1987, with phage doses ranging from 50 to  $10^{10}$  PFU and administration times ranging from pre- to postinfection, as well as from the continuous exposure via the environment (calf litter) to manage *E. coli* diarrhea (86). It should, however, be noted that in this study a nonenteropathogenic *E. coli* isolate and an uncharacterized *Lactobacillus* strain were always administered with the *E. coli* pathogenic strain targeted by the phages, without any information provided on whether or not phages were able to infect the nonenteropathogenic strain, which raises questions regarding the conclusions drawn by the authors. A solution to the degradation of phage particles during the intestinal transit may reside in the use of phages encapsulated in liposomes, polymers, or other formulations. Promising observations have been reported but need to be confirmed, and the kinetics of phage release in the gut must be thoroughly investigated (87, 88). Finally, recent data from a mammalian cell model showed that the gut epithelium internalizes virions, a phenomenon that could further reduce the number of available phages in the gut (89).

Nonetheless, once the phages have reached the gut section, where the target bacteria reside, these phages need to find a susceptible host. Indeed, the gut environment, such as the availability of nutrients or the oxygen concentration, among other factors, affects the bacterial physiology, which in turn could affect the susceptibility of bacteria to phages (90–92). Moreover, a pathogen will likely perturb the host physiology, which in turn could affect bacterial susceptibility to phages (Fig. 2) (93, 94). In addition, the spatial distribution of phages and bacteria in gut sections may be beneficial for one or the other population, promoting their coexistence, as has been recently shown *in vivo* (75). The property of some phages to bind to mucins would alter their diffusion but may increase their ability to reach bacteria embedded in this extracellular structure lying at the surfaces of epithelial cells (95, 96). Therefore, solutions to overcome the aforementioned challenges reside in the selection of phages with improved abilities to reach their target, which requires a preexisting knowledge of what is or are the specific behaviors of these targets in the gut.

Once they reach the surfaces of bacterial cells, phages have to defeat bacterial defense systems that drive the growth of phage-resistant bacteria. These defenses include mutations, surface modifications, restriction-modification, abortive infection, and CRISPR (clustered regularly interspaced short palindromic repeats) systems (Fig. 2). A variety of novel systems has rapidly developed during the past few years but, thankfully for phages, not every bacterium possesses all of them (28). Indeed, developing phage resistance can affect bacterial fitness, as shown by the reduced virulence of such phage-resistant bacteria in animal models of infection, which overall could be beneficial for patients treated with phages (97–100). In addition, most of the recently uncovered phage resistance systems do not provide a full phage resistance phenotype but instead decrease susceptibility by several logs, leaving opportunities for phages to amplify. Indeed, through ages of coevolution with bacteria, phages defeated these defense systems by evolving genetic variants, modifying their nucleotidic bases, or even inventing new functions, such as anti-CRISPR proteins (Fig. 2). Reciprocally for bacteria, not all phages possess the ability to develop a large variety of counter-resistance measures. It has also been shown that phages can jump from susceptible to resistant bacteria in the mouse gut by a single mutation in their tail fibers, which demonstrates that phages can rather quickly adapt to fluctuating bacterial populations in order to persist in this organ (101). To conclude, every phage-bacterium combination possesses its own potential for coevolution; this highly complicates the choice of phages and their combinations (phage cocktails) for therapeutic applications.

Finally, another layer of complexity is provided by temperate phages hidden within bacterial genomes. Their abundance in the gut has been shown to be higher in IBD patients compared to healthy adults (12). This abundance is necessarily linked to phage activity, i.e., their excision from bacteria. Some factors, such as diet and inflammation, induce prophage excision in the gut, but many remain to be identified, as well as the molecular cascade involved (102, 103). Lysed bacteria upon prophage induction will increase bacterial debris to which virulent phages could bind, and this may lower the dose of available phages for therapeutic interventions. Likewise, when both temperate and virulent phages recognize the same receptor, they could compete with each other. It has also been reported in a study of model phages (lambda and T4) that lysogens, bacteria carrying prophages, prevent the infection of bacteria by expressing defense systems against virulent phages (abortive infection) (104). In addition, hom immunity prevents lysogens from being infected by closely related temperate phages (105). Nevertheless, temperate phages may also be used as a solution when virulent phages are not readily isolated. Their genetic modifications, while inserted in the bacterial chromosome, are accessible in order to make them virulent and therefore more suitable for applications, as shown in animal models of *C. difficile* infections or, recently, during the compassionate treatment of a *Mycobacterium abscessus* infection in a cystic fibrosis patient (34, 106). Overall, both virulent and temperate phages will face the same challenges in the gut, with perhaps an advantage to the temperate phages, since during their stay within intestinal bacteria they may acquire functions useful to survive in this environment.

## CONCLUSIONS

During billions of years of evolution, phages have infected bacteria in different environments, and the guts of mammals are certainly not an exception. Therefore, the lack of success in using phages in therapeutic interventions in the gut reflects more our limited knowledge of this ecosystem than the intrinsic capacity of phages to infect intestinal bacteria. In particular, there is a very poor mechanistic appreciation of the factors that govern phage activity in the gut. The multiple experimental models examined here clearly show a breadth of possibilities rather than merely highlighting a path to success. In order to improve our knowledge obtained from animal models, we recommend that future studies incorporate the following: (i) timely quantification of viable phage particles supporting phage amplification; (ii) monitoring of phage resistance over time and treatments; (iii) avoidance of models for which the bacterial transit time is lower than 72 h, unless it reflects intestinal acute infections; and (iv) administration of phages at least 4 h after the bacterial challenge to allow bacteria to adapt to the intestinal environment, unless pretreatment (at least 4 h ahead of the bacterial challenge) is being evaluated.

Future research in this area will require bridging data from metagenomic studies with simplified experimental models. One possible way to progress in this direction would be to build increasingly complex synthetic systems, such as those based on gnotobiotic/isobiotic murine models (107). Although still imperfect in several ways, these models offer a very high reproducibility backbone that would help in addressing specific questions regarding molecular mechanisms. Nevertheless, the most convincing intestinal phage therapy data examined here arose from models in which an intestinal disease was ongoing, which is directly in line with the growing list of successful compassionate treatments currently provided to patients facing therapeutic inefficacy of antibiotic treatments. This argues for the necessity to consider the host, in addition to bacteria and phages, in identifying the conditions required for optimal efficacy of phage applications in the gut.

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### 3.4. Compléments de la revue de la littérature

Ce travail était initialement une revue systématique de la littérature qui a été menée selon les lignes directrices du PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses).(48)

#### *3.4.1. Critères d'inclusion*

Type d'études et d'interventions : les études interventionnelles publiées en anglais ont été incluses sans restriction de date.

Résultats et population : nous avons inclus des études expérimentales réalisées chez des mammifères et évaluant l'efficacité de l'administration de phages ciblant les bactéries provoquant une infection ou une colonisation digestive.

#### *3.4.2. Critères d'exclusion*

Nous avons exclu les modèles utilisant les oiseaux car leur microbiote intestinal diffère significativement de celui des mammifères.(47)

Ont également été exclus : les études in vitro, les modèles d'infection non-digestive tels que les péritonites ou les septicémies, l'absence d'infection ou de colonisation digestive expérimentale, l'utilisation de protéines phagiques, l'utilisation de phages uniquement pour moduler le microbiote intestinal sans infection ni colonisation par une bactérie spécifique ainsi que l'absence d'administration de phages.

#### *3.4.3. Méthodes de recherche*

Nous avons effectué nos recherches dans les bases de données suivantes en avril 2020 :

- Le registre central Cochrane des essais contrôlés (CENTRAL) sur la

bibliothèque Cochrane

- MEDLINE (via Pubmed)
- EMBASE (via Ovid)
- La base de données Française PASCAL de l'Institut d'information scientifique et technique

Nous avons vérifié les références énumérées de tous les articles inclus et effectué des recherches dans les bases de données des journaux correspondants. Nous avons également recherché la littérature grise via OpenGrey. Nous avons mis à jour ces recherches en Avril 2021. Nos algorithmes de recherche pour les différentes bases de données comprenaient les termes suivants : (phage, bactériophage) et (tractus gastro-intestinal, intestin, microbiome gastro-intestinal, microbiome, microbiote, flore intestinale).

#### *3.4.4. Sélection des articles*

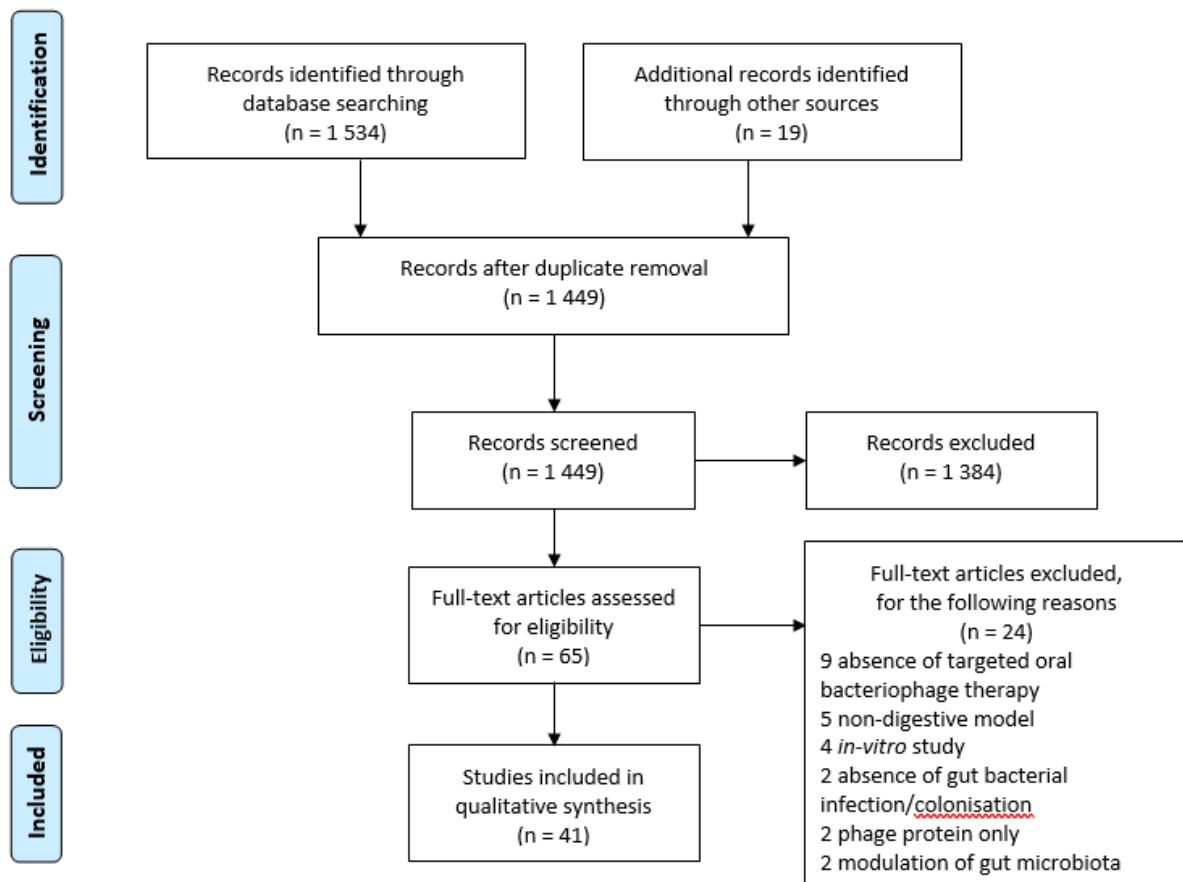
Trois examinateurs indépendants (François Javaudin, Chloé Latour et Quentin Lamy-Besnier) ont sélectionné les titres, les résumés puis les textes intégraux en respectant les critères d'inclusion et d'exclusion. Les divergences ont été résolues par consensus (n = 2).

#### *3.4.5. Résultats de la recherche d'articles*

Cette recherche a donné 1091 résultats dans MEDLINE, 264 résultats dans EMBASE, 25 résultats dans le Registre Central Cochrane des essais contrôlés (CENTRAL), 20 résultats dans la base de données PASCAL et 5 résultats dans OpenGrey. Une mise à jour a été effectuée en avril 2021 et comprenait 129 références supplémentaires. Dix-neuf articles supplémentaires ont été identifiés par d'autres sources (connaissance des auteurs, n = 3 ; à partir des références bibliographiques des

articles sélectionnés,  $n = 16$ ). Après avoir supprimé les doublons, 1449 articles ont été analysés et soumis aux critères de sélection, ce qui a abouti à un ensemble final de 41 articles sélectionnés (Figure 8).

Les études ont été publiées entre 1963 et 2020 et se sont réparties entre 9 pathogènes : *Clostridium difficile* ( $n = 3$ ), *Enterococcus faecalis* ( $n = 1$ ), *Escherichia coli* ( $n = 22$ ), *Listeria monocytogenes* ( $n = 1$ ), *Pseudomonas aeruginosa* ( $n = 1$ ), *Salmonella typhimurium* ( $n=7$ ), *Shigella sonnei* ( $n = 1$ ), *Vibrio cholerae* ( $n = 4$ ) et *Yersinia enterocolitica* ( $n = 1$ ). La plupart de ces études ont été réalisées sur des souris et des porcs et, dans une certaine mesure, sur des veaux, des hamsters, des agneaux, des lapins, des rats, des moutons et des bœufs.



**Figure 8.** Sélection des articles de la revue systématique de la littérature.

# Méthodes et résultats

Les méthodes et les principaux résultats sont résumés dans l'article publié dans la revue *Microorganisms* (IF 2020 = 4.128) en décembre 2021.

1. Article : "Impact of Phage Therapy on Multidrug-Resistant *Escherichia coli* Intestinal Carriage in a Murine Model"



## Impact of Phage Therapy on Multidrug-Resistant *Escherichia coli* Intestinal Carriage in a Murine Model

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## Abstract

**Introduction:** The growing resistance of bacteria to antibiotics is a major global public health concern. An important reservoir of this resistance is the gut microbiota. However, limited data are available on the ability of phage therapy to reduce the digestive carriage of multidrug-resistant bacteria. **Materials and methods:** Four novel lytic phages were isolated in vitro for efficacy against an extended-spectrum beta-lactamase-producing (ESBL) *Escherichia coli* strain also resistant to carbapenems through a carbapenemase OXA-48. The first step was to develop models of ESBL *E. coli* digestive carriage in mice. The second step was to test the efficacy of an oral and rectal phage therapy (a cocktail of four phages or microencapsulated phage) to reduce this carriage. **Results:** The two most intense models of digestive carriage were obtained by administering amoxicillin ( $0.5 \text{ g}\cdot\text{L}^{-1}$ ) continuously in the drinking water (Model 1) or pantoprazole ( $0.1 \text{ g}\cdot\text{L}^{-1}$ ) continuously in the drinking water, combined with amoxicillin ( $0.5 \text{ g}\cdot\text{L}^{-1}$ ), for the first 8 days (Model 2). Oral administration of the phage cocktail to Model 1 resulted in a transient reduction in the concentration of ESBL *E. coli* in the faeces 9 days after the bacterial challenge (median =  $5.33 \times 10^8$  versus  $2.76 \times 10^9 \text{ CFU}\cdot\text{g}^{-1}$ ,  $p = 0.02$ ). In contrast, in Model 2, oral or oral + rectal administration of this cocktail did not alter the bacterial titre compared to the control (area under the curve, AUC,  $3.49 \times 10^9$ ;  $3.41 \times 10^9$  and  $3.82 \times 10^9$  for the control, oral and oral + rectal groups, respectively;  $p$ -value  $> 0.8$  for each two-by-two group comparison), as well as the administration of an oral microencapsulated phage in Model 1 (AUC =  $8.93 \times 10^9$  versus  $9.04 \times 10^9$ ,  $p = 0.81$ ). **Conclusions:** Oral treatment with amoxicillin promoted digestive carriage in mice, which was also the case for the addition of pantoprazole. However, our study confirms the difficulty of achieving efficacy with phage therapy to reduce multidrug-resistant bacterial digestive carriage in vivo.

*Keywords:* [phage therapy](#); [extended-spectrum beta-lactamase](#); [drug resistance](#); [multidrug-resistant bacteria](#); [intestinal carriage](#); [enterobacteriaceae](#); [Escherichia coli](#)

## 1. Introduction

The rise of bacterial resistance to antibiotics is considered a major public health concern worldwide. The World Health Organisation (WHO) has identified research on third-generation cephalosporins (3GC) and carbapenem-resistant *Enterobacteriaceae* as a key priority [1]. The gut microbiome is a major reservoir of antimicrobial resistance [2,3]. However, the healthy gut microbiota, under homeostatic conditions, can prevent colonisation by pathogens [4], but in the case of dysbiosis, induced by antibiotics, for example, intestinal colonisation by antibiotic-resistant agents is favoured [2]. Although this digestive carriage is asymptomatic, subjects colonised with extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-E) have a higher risk of developing an ESBL-E infection with increased morbidity, mortality, length of hospital stay and health care costs [5,6,7]. Furthermore, subjects with multidrug-resistant *Enterobacteriaceae* (MDR-E) intestinal carriage can contaminate their environment and thus can pass the resistant bacteria to others, especially in the hospital setting [8].

Different digestive decontamination strategies have been evaluated, but none have proved useful in routine practice [9]. However, new strategies could be interesting, such as phage therapy, but evidence is still lacking [10]. Oral phage therapy is considered safe in humans and has many advantages (target specificity, bactericidal activity, low environmental impact) [11,12]. Moreover, the mechanisms of bacterial resistance to antibiotics and phages are completely different, which means that a high level of antibiotic resistance in a bacterium is not necessarily associated with phage resistance [13,14]. Some animal studies have

investigated the effect of phage therapy on intestinal carriage of *Enterobacteriaceae*, but one have been conducted on MDR-E [15].

Therefore, the aim of our study was to evaluate the ability of phage therapy to reduce the digestive carriage of MDR-E in a murine model.

## 2. Materials and Methods

### 2.1. Animals and Ethics Statement

Mice (6-week-old male C57BL/6J mice) provided by the Janvier laboratory (Le Genest-Saint-Isle, France) were used. The mice were housed at the Animal Research Centre of the Institute of Health Research 2, University of Nantes, and fed a controlled sterile diet in a controlled environment (12-h day/night cycle). Drinking water and food were provided ad libitum. The animals were isolated in individual cages with environmental enrichment to avoid cross-contamination by coprophagy. Their well-being was monitored daily.

The study was approved by the French Ministry of Higher Education and Research (APAFIS 11056) and by the Animal Ethics Committee of the Health Research Institute (reference 201708291549991).

### 2.2. Bacterial Strains and Gastric Gavage

A clinical strain of ESBL-producing *Escherichia coli* (*E. coli*), also resistant to carbapenems through a carbapenemase OXA-48, isolated from a surgical wound, was used to induce intestinal colonisation. Bacterial challenge was performed using 20-GA plastic feeding tubes (FTP-20-38, Instech Laboratories, Plymouth Meeting, PA, USA) and injecting 200  $\mu$ L of solution into the gastric contents, containing a total of  $10^6$  CFU of ESBL *E. coli*.

### 2.3. Phages and Gastric-Rectal Administration

The phages were isolated and selected by the Clean Cells company (Montaigu, France).

Microencapsulation of the PEC02 phage was performed by Kerry Richards and Danish J. Malik of the Department of Chemical Engineering of Loughborough University, Loughborough, UK, using a scalable membrane emulsification process [16].

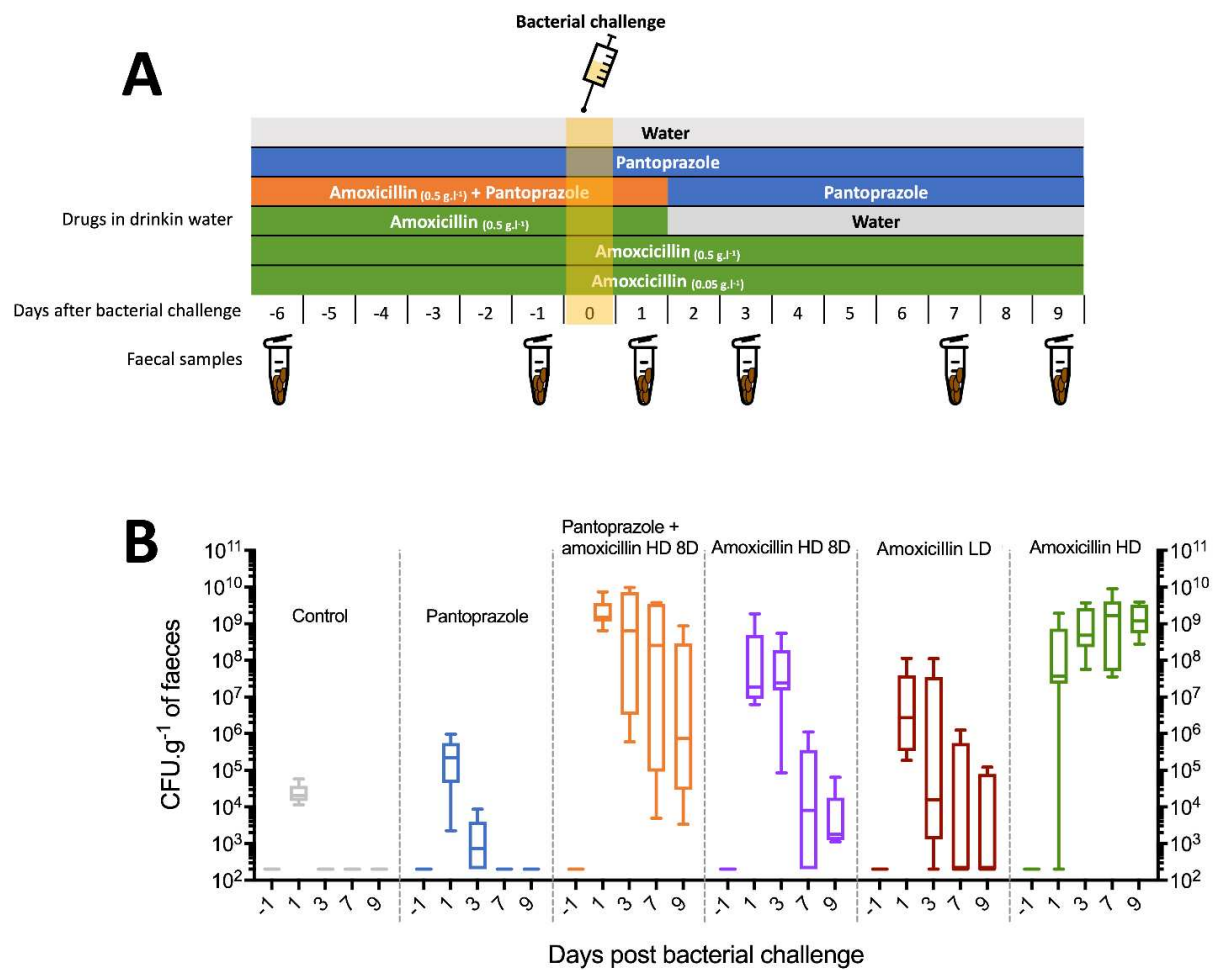
Gastric administration was performed using 20 GA (Instech Laboratories, USA) or 18-GA (Instech Laboratories, Plymouth Meeting, PA, USA) plastic feedings tubes for encapsulated phages, followed by the injection of 200  $\mu\text{L}$  of a solution containing a cocktail of the four phages ( $2 \cdot 10^8 \text{ PFU} \cdot \text{mL}^{-1}$ ) or PEC02 microencapsulated phages ( $1 \cdot 10^6 \text{ PFU} \cdot \text{mL}^{-1}$ ) into the gastric contents.

Rectal administration was performed using a 24-GA intravenous catheter (Terumo, Tokyo, Japan) and injecting 100  $\mu\text{L}$  of solution containing a cocktail of the four phages ( $2 \cdot 10^8 \text{ PFU} \cdot \text{mL}^{-1}$ ) into the rectal contents. After rectal administration, mice were held in an inverted position for 30 s.

#### *2.4. Murine Model of Intestinal Colonisation*

Mice were exposed to different treatment regimens to induce intestinal dysbiosis. We used amoxicillin at concentrations of  $0.5 \text{ g} \cdot \text{L}^{-1}$  (high dose, HD) or  $0.05 \text{ g} \cdot \text{L}^{-1}$  (low dose, LD) and pantoprazole at  $0.1 \text{ g} \cdot \text{L}^{-1}$ , according to the schemes summarised in [Figure 1](#), all in drinking water. We used injectable forms of these drugs in order to ensure that they were soluble in the water. Briefly, we tested six conditions: water, pantoprazole, pantoprazole + amoxicillin HD for the first 8 days, amoxicillin HD for the first 8 days, amoxicillin HD and amoxicillin LD throughout the experiment. The bottles were refilled every 3 days. There were six mice per condition tested. Bacterial challenge was performed after 7 days from the start of amoxicillin and/or pantoprazole. Stool sampling was performed at days 1, 6, 8, 10, 14 and 16.





**Figure 1.** Murine model of ESBL *E. coli* intestinal colonisation (6 mice per group) (A), Experimental protocol. (B), Faecal concentration of ESBL *E. coli*.

### 2.5. Faecal Collection and Culture

On the day of sampling, each mouse was placed in a clean cage for 1 h to collect its faeces, which were immediately frozen at  $-80\text{ }^{\circ}\text{C}$ . Each stool was weighed and then crushed (Mixer Mill MM 400, Retsch, Haan, Germany) with 1 mL of sterile saline water. Serial dilutions were performed, followed by manual inoculations of 100  $\mu\text{L}$  onto ESBL selective chromogenic agar plates (ChromID ESBL, BioMérieux, Marcy-l'Étoile, France) before incubation at  $37\text{ }^{\circ}\text{C}$  for 24 h. ESBL *E. coli* were

identified by the pink to burgundy coloration they take on these specific agar plates ([Figure S1 in the Data Supplement](#)).

## 2.6. *In-Vivo* Evaluation of Phage Therapy

### 2.6.1. Experiment 1

Mice were exposed to amoxicillin treatment in the drinking water ( $0.5 \text{ g}\cdot\text{L}^{-1}$ ) throughout the experiment. Bacterial challenge was performed on day 7. From day 9 to day 11, six mice were treated daily by gastric gavage with the phage cocktail or by gastric gavage of  $200 \mu\text{L}$  of water for the control group ( $n = 6$ ). Faecal samples were collected on days 1, 6, 8, 10, 14, 16 and 18.

### 2.6.2. Experiment 2

Throughout the experiment, the drinking water contained  $0.5 \text{ g}\cdot\text{L}^{-1}$  of amoxicillin. Bacterial challenge was performed on day 7. The microencapsulated phage (PEC02) was administered by gastric gavage on days 7, 8 and 9 in eight mice (control group,  $n = 4$ ). Faecal samples were collected on days 1, 6, 8, 10, 12, 16 and 18.

### 2.6.3. Experiment 3

Mice were exposed to amoxicillin treatment in the drinking water ( $0.5 \text{ g}\cdot\text{L}^{-1}$ ) for the first 8 days. In addition, the drinking water contained pantoprazole ( $0.1 \text{ g}\cdot\text{L}^{-1}$ ) throughout the experiment to facilitate intestinal colonisation and limit phage destruction during gastric transit [[17,18](#)]. Bacterial challenge was performed on day 7. From day 14 to 18, mice were treated daily with the phage cocktail by gastric gavage (oral group,  $n = 8$ ) or by gastric and rectal route (oral + rectal group,  $n = 8$ ) or by gastric gavage of  $200 \mu\text{L}$  water for the control group ( $n = 8$ ). Stool samples were taken on days 1, 6, 8, 10, 14 and every 2 days until day 24.

### 2.6.4. Statistical Analysis

Statistical analyses were performed using the R software (R Foundation for Statistical Computing, Vienna, Austria) version 4.0.0 and GraphPad Prism 8.2.1 (GraphPad Software Inc., San Diego, CA, USA). Areas under the curve (AUC) were calculated by the trapezoidal method and compared with a Kruskal-Wallis test. Two-way repeated measures (RM) ANOVA was performed to compare multiple groups, and non-parametric Mann-Whitney tests were performed between groups two-by-two. Negative cultures were fixed at  $2 \log_{10}$  CFU·g<sup>-1</sup> of faeces (mean faeces mass was approximately 50 mg). All tests were defined with an alpha risk determined a priori as significant if 0.05.

### 3. Results

#### 3.1. Isolation and Selection of Phages

Out of 20 screened phages, four lytic phages (PEC02, PEC08, PEC16, PEC18) were isolated and selected for their specific activity in vitro against the ESBL *E. coli* used. When all four were used together in a cocktail they totally inhibited bacterial growth under these in vitro conditions ([Figures S2 and S3 in the Data Supplement](#)).

#### 3.2. Development of the Murine Model of ESBL *E. coli*

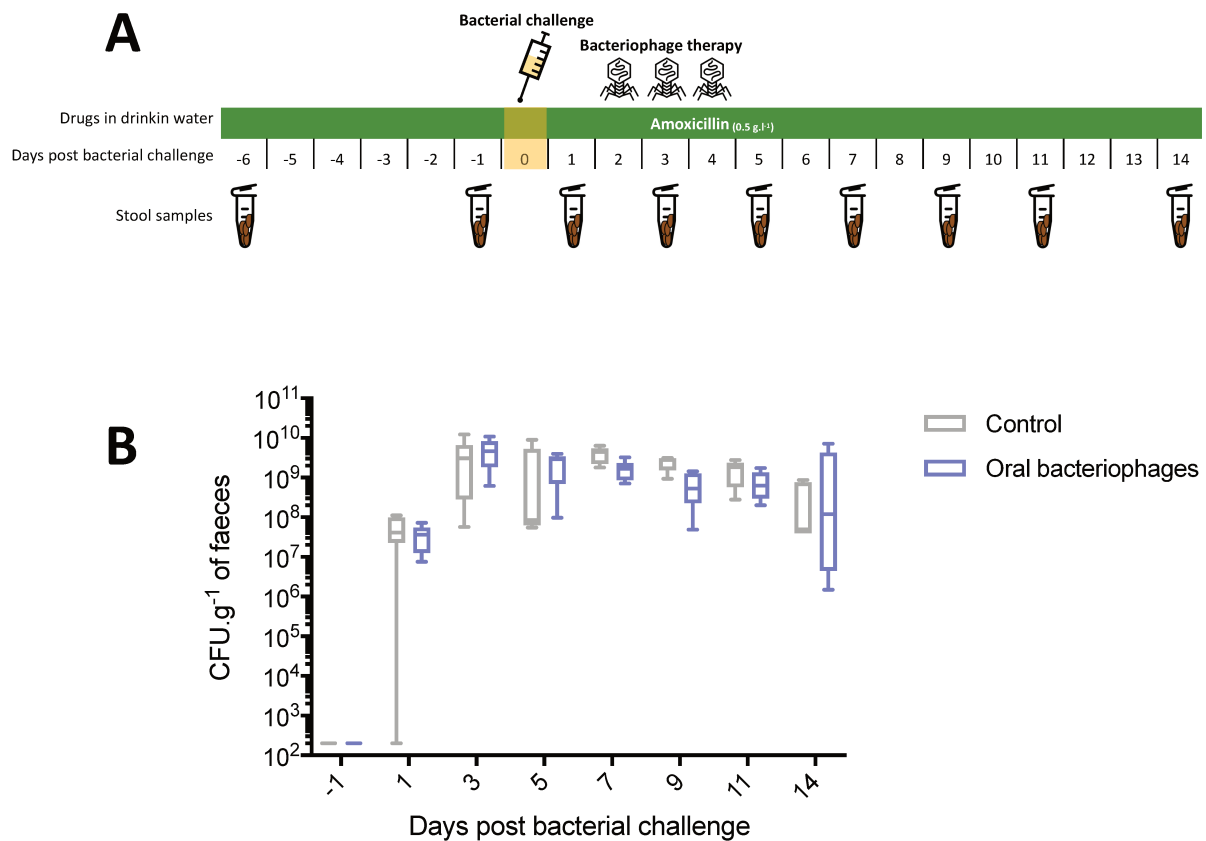
The experimental designs of the different groups are shown in [Figure 1A](#). The experimental regimen with continuous administration of high-dose amoxicillin (HD) and pantoprazole + amoxicillin HD 8D resulted in higher colonisation (median AUC =  $1.09 \times 10^{10}$  and  $1.39 \times 10^{10}$  CFU·g<sup>-1</sup>·day, respectively;  $p = 0.70$  and  $p < 0.05$  compared to the other groups). Continuous administration of amoxicillin HD resulted in lower inter-individual variability between days 3 and 9, with a higher faecal concentration at day 9 ([Figure 1B](#)). The addition of pantoprazole resulted in an increase in the initial faecal concentration (day 1 after bacterial challenge) of ESBL *E. coli* compared to amoxicillin HD 8D alone (median =  $1.52 \times 10^9$  versus  $3.25 \times 10^7$  CFU·g<sup>-1</sup>;  $p = 0.007$ ). This effect was also still present at day 9 (median =  $7.45 \times 10^5$  versus  $1.81 \times 10^3$  CFU·g<sup>-1</sup>;  $p = 0.009$ ), and the median AUC was higher ( $1.39 \times$

$10^{10}$  versus  $8.83 \times 10^7$  CFU·g<sup>-1</sup>·day;  $p = 0.009$ ). However, there was no statistical difference in colonisation between the control and pantoprazole groups (median AUC =  $3.06 \times 10^4$  versus  $3.33 \times 10^5$ ;  $p = 0.06$ ).

### 3.3. *In Vivo* Evaluation of Phage Therapy

#### 3.3.1. Experiment 1: Oral Cocktail of Phages

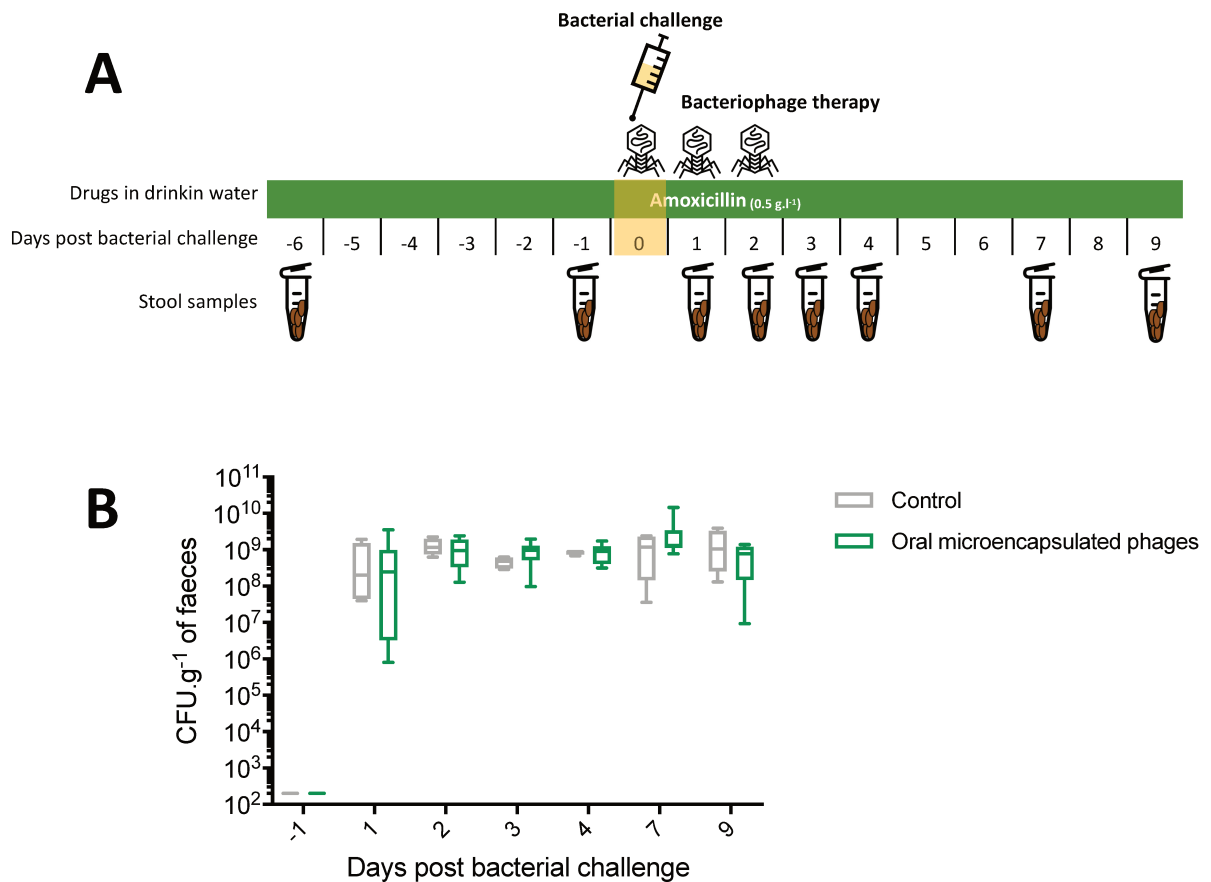
[Figure 2A](#) summarises the experimental procedure. In this experiment, we observed one death in the control group. There was no significant difference in the faecal ESBL *E. coli* concentration between the two groups when total time was taken into account (median AUC =  $2.82 \times 10^{10}$  in the control group versus  $2.54 \times 10^{10}$  in the treated group,  $p = 0.54$ ; 2-way RM ANOVA,  $p = 0.51$ ). However, in the phage-treated group, we measured a transient decrease in this faecal concentration, starting on days 7 to 11 after bacterial challenge ([Figure 2B](#)). This effect was statistically significant on day 9 (median =  $5.33 \times 10^8$  versus  $2.76 \times 10^9$  CFU·g<sup>-1</sup>,  $p = 0.02$ ).



**Figure 2.** Experiment 1: oral cocktail of phages (A), Experimental protocol. (B), Faecal concentration of ESBL *E. coli*. Control group ( $n = 6$ ), oral bacteriophages group ( $n = 6$ ).

### 3.3.2. Experiment 2: Microencapsulated Phages

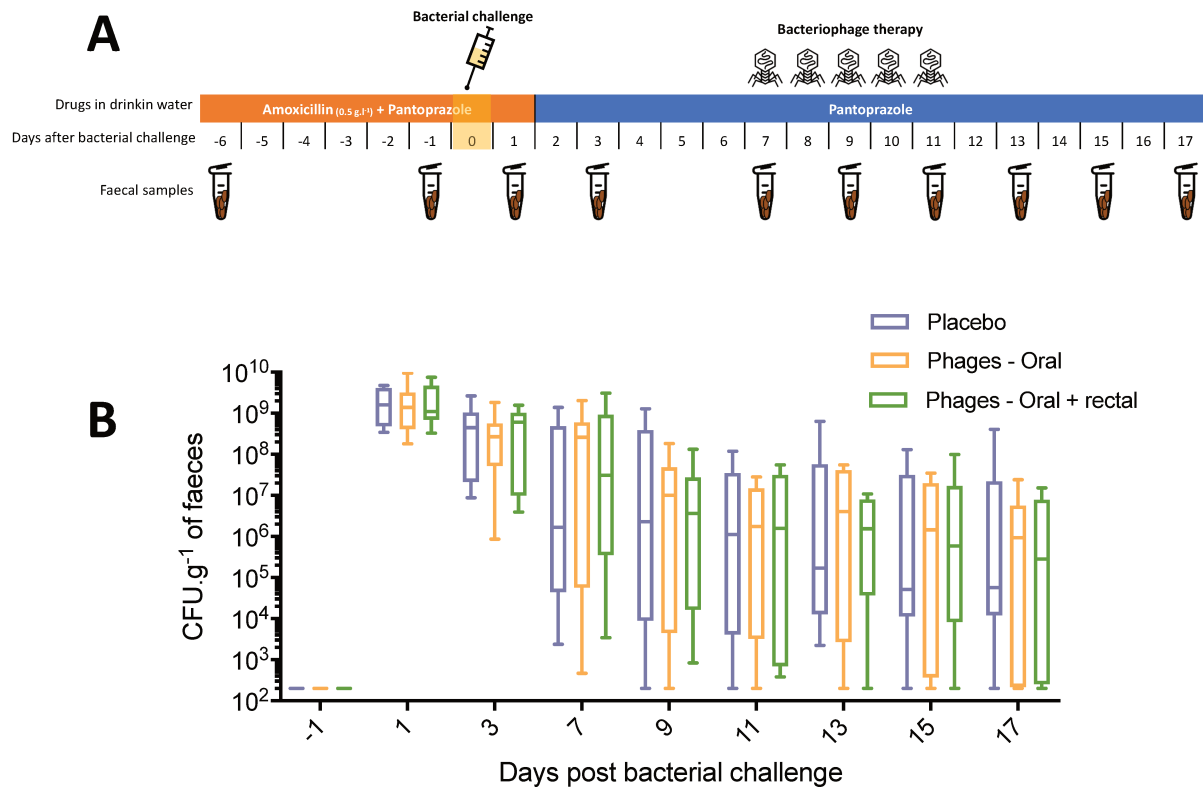
The experimental design is illustrated in [Figure 3A](#). There was no significant difference in faecal ESBL *E. coli* concentration between the control and the oral microencapsulated phage groups (median AUC =  $8.93 \times 10^9$  versus  $9.04 \times 10^9$  respectively,  $p = 0.81$ ; 2-way RM ANOVA,  $p = 0.44$ ). No transient treatment effect could be observed ([Figure 3B](#)).



**Figure 3.** Experiment 2: microencapsulated phages (A), Experimental protocol. (B), Faecal concentration of ESBL *E. coli*. Control group ( $n = 4$ ), oral microencapsulated phages group ( $n = 8$ ).

### 3.3.3. Experiment 3: Oral and Rectal Cocktail of Phages with Pantoprazole

The median and interquartile ranges of ESBL *E. coli* faecal concentrations are presented in [Figure 4B](#). We observed no differences in ESBL *E. coli* concentration between groups, neither for the area under the curve ( $3.49 \times 10^9$ ,  $3.41 \times 10^9$  and  $3.82 \times 10^9$  for the control, oral and oral + rectal groups, respectively;  $p$ -value  $> 0.8$  for each 2-by-2 group comparison) nor for the 2-way RM ANOVA ( $p$ -value = 0.96) or the 2-by-2 comparison (Mann Whitney  $p$ -value  $> 0.05$  for each point).



**Figure 4.** Experiment 3: oral and rectal cocktail of phages with pantoprazole (**A**), Experimental protocol. (**B**), Faecal concentration of ESBL *E. coli*. Placebo group ( $n = 8$ ), oral phages group ( $n = 8$ ), oral and rectal phages group ( $n = 8$ ).

## 4. Discussion

### 4.1. Summary of Results

In our study, we were not able to show the long-term efficacy of phage therapy in reducing this carriage. We measured a transient reduction in faecal concentration of approximately 1 log CFU·g<sup>-1</sup>. Nevertheless, we were able to develop several models of intestinal carriage of ESBL *E. coli* with the administration of drugs in drinking water. The advantage of our models was that beta lactam and PPI were diluted in drinking water, which is simpler than some previous models with subcutaneous injections [17,19,20].

#### *4.2. Effects of PPI on Digestive Colonisation*

We showed that the addition of PPI to antibiotic therapy not only increased the initial faecal ESBL concentration, as shown by Stiefel et al. [17], but also persisted at higher titres away from the bacterial challenge. This facilitating effect of colonisation persistence in our model fits well with observations on humans. Indeed, the meta-analysis of Willems et al. showed an association between the use of acid suppressants and the risk of colonisation by multidrug-resistant microorganisms (MDRO) [21]. The faecal microbiome of PPI users is altered with an over-representation of oral bacteria [22,23]. These alterations in the gut microbiome are the basis for continued MDRO colonisation, but further work is needed to support this hypothesis.

#### *4.3. Effectiveness and Limitations of Phages in Reducing Digestive Carriage*

In the literature, many phage models aimed at reducing *E. coli* carriage in mice have proven ineffective [15,18,24,25,26] or only transient [27,28,29], regardless of whether phage therapy is administered before or after bacterial challenge, with a single phage or a phage cocktail. The few models that showed efficacy had a treatment protocol that was not considerably different from that of other models without efficacy [15,30,31]. In our model with continuous pressure of antibiotic therapy, we did not observe a lasting efficacy of phage therapy. These results are in agreement with the experiments of Galtier et al. [15], who, in their continuous antibiotic pressure model, found no efficacy. In contrast, a single dose of a cocktail of three phages had good efficacy on the digestive carriage of uropathogenic *E. coli* when antibiotic pressure was no longer present [15]. Proton pump inhibitors also cause changes in the gut microbiota, which may have influenced our results since we did not deal with continuous antibiotic pressure but with continuous pressure by PPI.

The conditions for the optimal efficacy of phage therapy in the gastrointestinal tract are still poorly understood, requiring additional knowledge. Before phages can



successfully infect target bacteria in the gut, they must be able to reach and contact them [32]. However, the gut environment can affect bacterial physiology, which may reduce the efficacy of phages compared to in-vitro conditions [33,34,35]. Target bacteria may also reside in the mucosa, which is difficult for phages to access [36]. Another problem is the degradation of phages during intestinal transit and, especially, during gastric passage at low pH [11,18,25]. To counter this phenomenon, we used PPI to increase the gastric pH, in addition to micro-encapsulation of phages. Encapsulation effectively limits the reduction of phage concentration in the gastrointestinal tract after oral administration [37]. Indeed, phages are generally not stable in an environment so acidic as the stomach, some studies use a bicarbonate buffer with oral administration of phages [18,37]. However, the only study on *E. coli* (nalidixic acid-resistant O157:H7) that we found in the literature using encapsulated phages did not show in-vivo efficacy in a bovine model [38]. In contrast, *E. coli* appears to be able to survive for several days in an acidic environment [39]. A limitation of our work regarding the lack of evidence for the efficacy of microencapsulated phages is that we were only able to test it on one phage (PEC02), although this was the most active phage in vitro ([Figure in the Supplementary Data](#)). Other animal models have been developed with encapsulated phages to treat *Salmonella* infections, and the results are encouraging [40,41]. However, these infection models are somewhat different from the asymptomatic digestive colonisation models [32].

#### 4.4. Limitations of the Study

First, the phages used were sent to us directly by the Clean Cells company and the identification and selection data are only partially available. The precise methods used for in vitro manipulations are not available.

Second, in our study we did not measure the gastric pH or monitor the survival and possible amplification of phages in the digestive tract of mice.

Third, our colonisation model using continuous high dose amoxicillin results in a very high concentration of ESBL *E. coli* which probably reaches a saturation plateau. This model deviates from the colonisation level conditions of human pathology but has the advantage of being stable and homogeneous unlike other models tested where the carriage level was lower over time.

Fourth, a potential transfer of resistance to other bacteria was not specifically evaluated in our study.

Finally, we did not develop a positive model resulting in a significant reduction in ESBL *E. coli* carriage using, for example, antibiotics. Our aim was only to compare phage therapy to placebo.

#### *4.5. Perspectives*

Finally, phage therapy seems highly attractive for eliminating or reducing the carriage of multidrug-resistant bacteria in the digestive tract because of its specificity, its low impact on the digestive microbiome and its safety. However, the in-vivo results are, so far, rather dissatisfying, except for some infectious models such as cholera [32]. Moreover, the only human study of intestinal phage therapy showed disappointing results; this clinical trial that attempted to treat *E. coli* diarrhoea in children failed to achieve replication of intestinal phages and improved outcomes [42]. There are still many obstacles to be elucidated in order to be able to implement this therapy in routine practice.

## **5. Conclusions**

Oral treatment with amoxicillin was able to promote digestive carriage in mice, which was also the case for the addition of PPI. However, our study confirms the difficulty of achieving efficacy with intestinal phage therapy to reduce MDR bacterial carriage in vivo. Only a small transient effect could be observed in one experiment, while the other experimental conditions showed no effectiveness.

Many obstacles need to be further investigated and better understood before this approach can be used in routine practice in this indication.

## Supplementary Materials

The following are available online at <https://www.mdpi.com/article/10.3390/microorganisms9122580/s1>. Figure S1. ESBL E. coli on chromatic ESBL agar plates (pink to burgundy coloration). Figure S2. Susceptibility of ESBL E. coli to phages tested. Figure S3. Monitoring of ESBL E. coli growth by OD 600nm reading on a 96-well plate automat.

## Author Contributions

Conceptualization, E.B. and E.M.; methodology, F.J., E.B. and E.M.; software, F.J. and E.B.; validation, P.B., E.B. and E.M.; formal analysis, F.J. and E.B.; investigation, F.J. and E.M.; resources, P.B.; data curation, F.J. and E.B.; writing—original draft preparation, F.J.; writing—review and editing, P.B., E.B. and E.M.; visualization, F.J.; supervision, P.B., E.B. and E.M.; project administration, E.B. and E.M.; funding acquisition, E.B. and E.M. All authors have read and agreed to the published version of the manuscript.

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## Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the French Ministry of Higher Education and Research (APAFIS 11056; 18 December 2017) and by the Animal Ethics Committee of the Health Research Institute (reference 201708291549991; 18 December 2017).

## Informed Consent Statement

Not applicable.

## Data Availability Statement

The datasets generated during and/or analysed during this study are available from the corresponding author upon reasonable request.

## Acknowledgments

Thanks to Axel Désir-Vigné and Régis Frénais for their help during the experiments.

## Conflicts of Interest

The authors declare that they have no conflict of interest.

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## Data Supplement



Figure S1. ESBL *E. coli* on chromatic ESBL agar plates (pink to burgundy coloration)

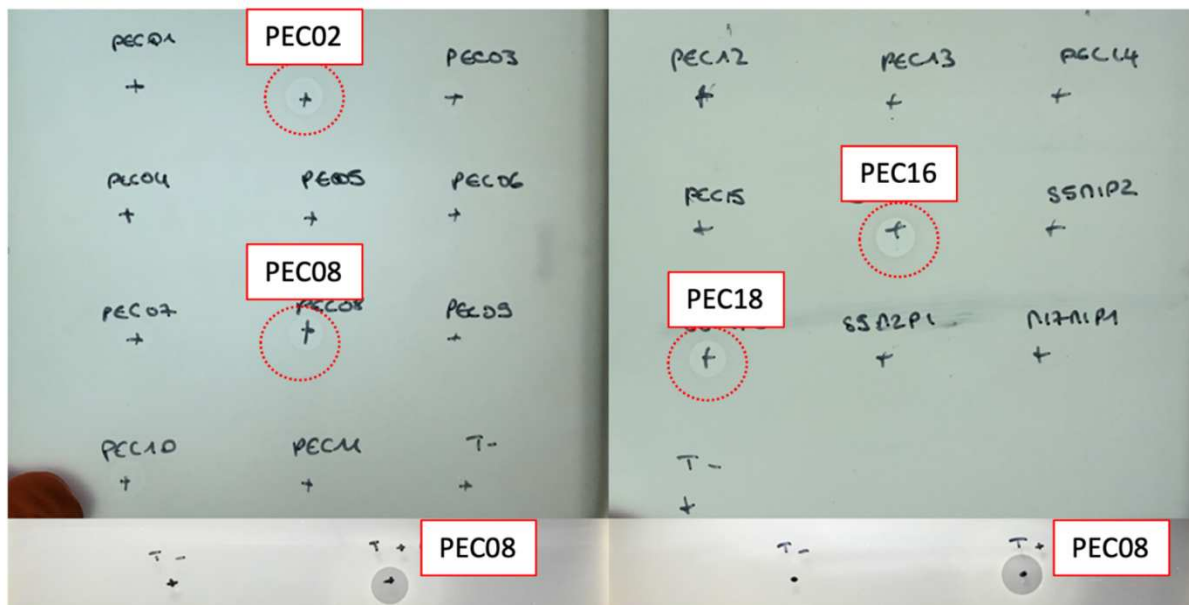


Figure S2. Susceptibility of ESBL *E. coli* to phages tested

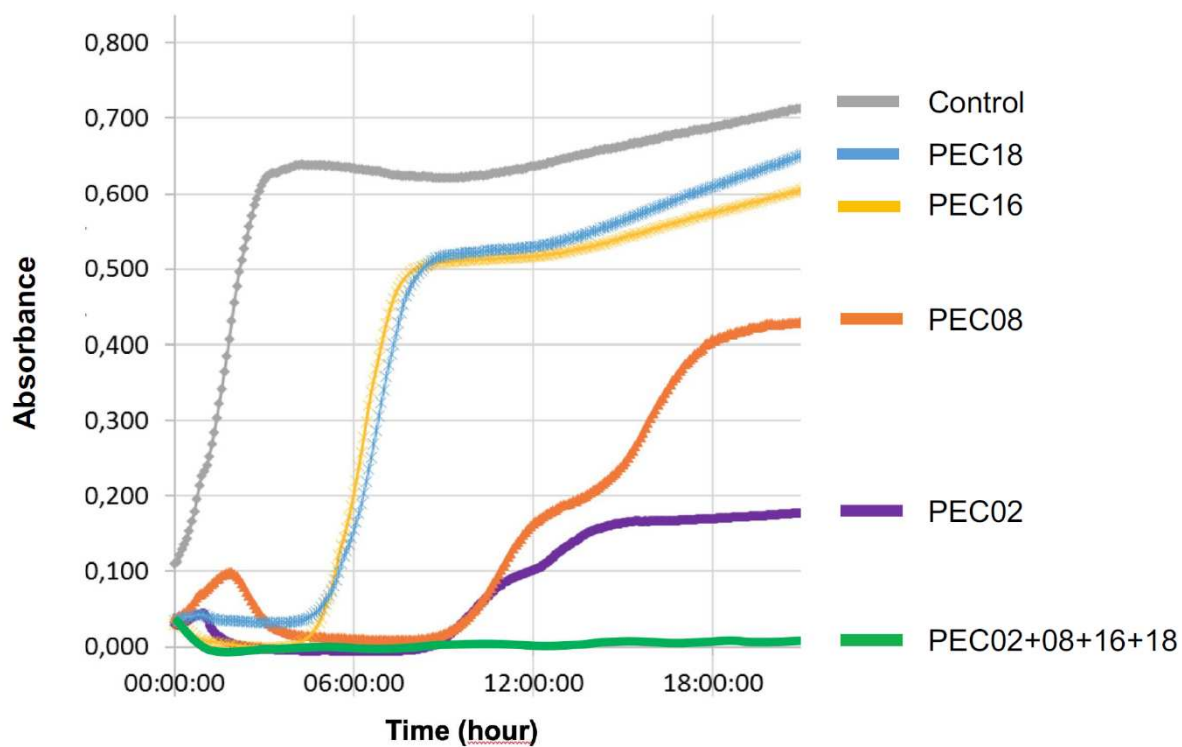


Figure S3. Monitoring of ESBL *E. coli* growth by OD 600nm reading on a 96-well plate automat.

## 2. Résultats complémentaires

### 2.1. Antibiogramme de l'*E. coli* sélectionné

L'*E. coli* choisi présentait une BLSE avec une carbapénèmase OXA 48.

Son antibiogramme est résumé dans le tableau 1.

**Tableau 1.** Antibiogramme de la souche d'*E. coli* choisi pour les expériences.

Antibiotique	CMI	
Ampicilline	> 16	R
Amoxicilline/acide clavulanique	> 16	R
Pipéracilline/tazobactam	> 64	R
Ticarcilline	> 64	R
Ertapénème	2	R
Imipénème	2	S
Céfotaxime	> 32	R
Ceftazidime	4	I
Tobramycine	≤ 1	S
Gentamicine	≤ 1	S
Amikacine	≤ 1	S
Acide nalidixique	> 16	R
Ofloxacine	> 4	R
Ciprofloxacine	> 2	R
Triméthoprim/sulfaméthoxazole	> 160	R
Nitrofurantoïne	32	S

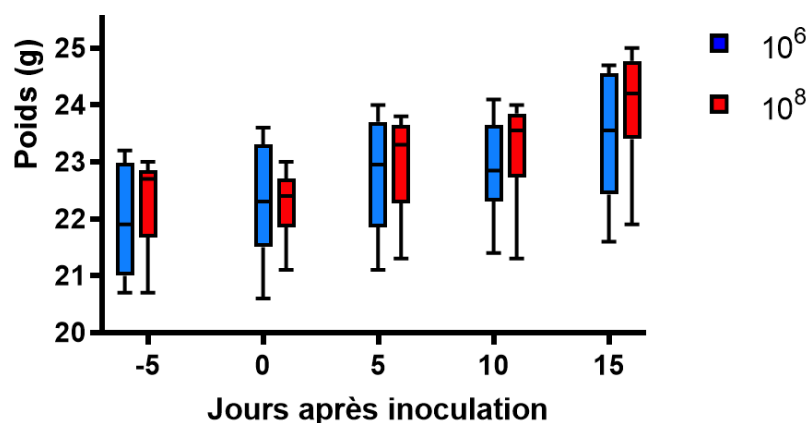
## 2.2. Influence de l'inoculum bactérien in vivo

### 2.2.1. Méthodes

Douze souris C57BL/6J ont été exposées à huit jours de traitement par amoxicilline diluée dans l'eau de boisson à la concentration de 0,5 g/L. Les biberons étaient renouvelés tous les trois jours. Au 7<sup>ème</sup> jour les souris recevaient l'inoculum bactérien par *E. coli* BLSE par gavage gastrique d'une solution de 200  $\mu$ L correspondant à un inoculum soit de  $10^6$  UFC pour un groupe (n = 6 souris) soit de  $10^8$  UFC pour l'autre groupe (n = 6 souris). Les selles ont été récoltées et mises en culture (cf « Materials and methods » de l'article ci-dessus) à j-6, j0, j1, j3, j6, j10 et j15 post inoculation. Le poids des souris était également relevé tous les 5 jours.

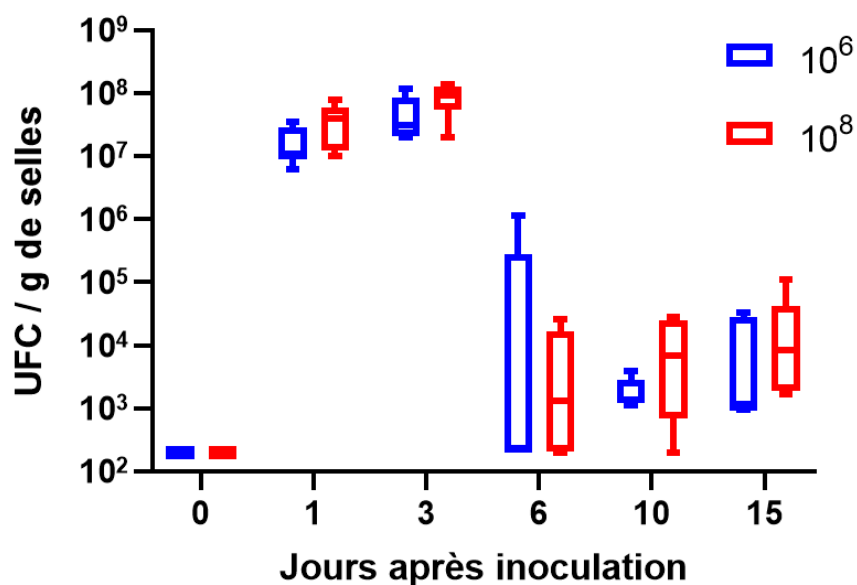
### 2.2.2. Résultats

Il n'y avait pas de différence significative ( $p > 0,05$  pour chaque point en analyse univariée) de poids entre les deux groupes (inoculum bactérien à  $10^6$  contre  $10^8$  UFC) au cours de cette expérience (Figure 8).



**Figure 8.** Poids des souris en fonction de l'inoculum bactérien ( $10^6$  UFC en bleu et  $10^8$  UFC en rouge).

Nous n'observons pas de différences de concentration du titre fécal en *E. coli* BLSE entre les deux groupes (Figure 9 ;  $p > 0,05$  pour chaque point en analyse univariée ;  $p = 0,15$  RM 2-way ANOVA ;  $p = 0,13$  en comparant les AUC).



**Figure 9.** Concentration fécale en *E. coli* BLSE selon l'inoculum bactérien ( $10^6$  UFC en rouge et  $10^8$  en bleu).

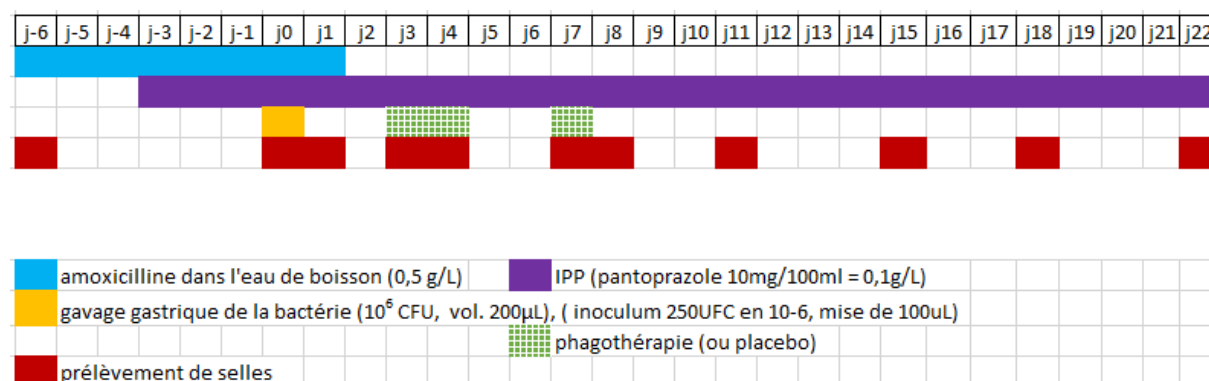
### 2.3. Phagothérapie séquentielle (2 + 1 jours) avec IPP en continu

#### 2.3.1. Méthodes

Douze souris C57BL/6J ont été séparées en deux groupes (phagothérapie et placebo) puis ont été exposées à huit jours de traitement par amoxicilline à la concentration de 0,5 g/L dans l'eau de boisson de j-6 à j1 post inoculation et au pantoprazole à la concentration de 0,1 g/L dans l'eau de boisson également de j-3 à j22 (Figure 10).

Les selles ont été récoltées et mises en culture (cf « Materials and methods » de l'article ci-dessus) à j-6, j0, j1, j3, j4, j7, j8, j11, j15, j18 et j22.

Les différents bactériophages utilisés dans l'expérience n°3 (PEC02, PEC08, PEC16 et PEC18) ont été dosés après congélation à -80°C et envoyés à la société CleanCells pour les j8, j11 et j18 du groupe de souris traité par phages (n = 6).



**Figure 10.** Schéma du protocole de l'expérience 4 (n = 6 par groupe).

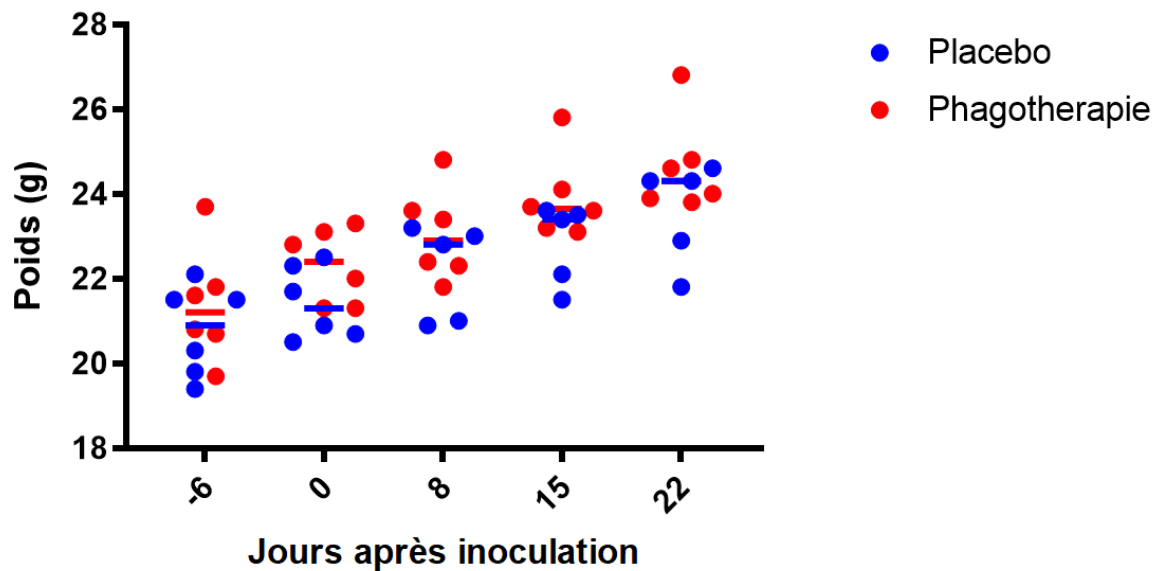
Les concentrations fécales des médicaments utilisés (pantoprazole et amoxicilline) ont été dosés par le Pr. Éric Dailly (Service de pharmacologie clinique, CHU Nantes) à l'aide d'un système de chromatographie (H-Class® Acquity UPLC system, Waters™, St Quentin en Yvelines, France) pour les souris du groupe contrôle (n = 6). Le système se composait d'une colonne Kinetex 2,6 µm C18 dans un four thermostaté à 50 °C, de phases mobiles avec un gradient binaire à un débit de 0.8 mL/min et un spectromètre de masse contrôlant les rapports m/z.

### 2.3.2. Résultats

Une souris du groupe contrôle est morte à j2 en raison d'une probable inhalation de la bactérie *E. coli* BLSE lors du gavage gastrique à j0.



Le poids des souris n'étaient pas différents entre les deux groupes au cours de l'expérience ( $p > 0,05$  pour chaque point ; Figure 11).



**Figure 11.** Poids des souris au cours de l'expérience 4 (en rouge le groupe traité par bactériophage, en bleu le groupe contrôle).

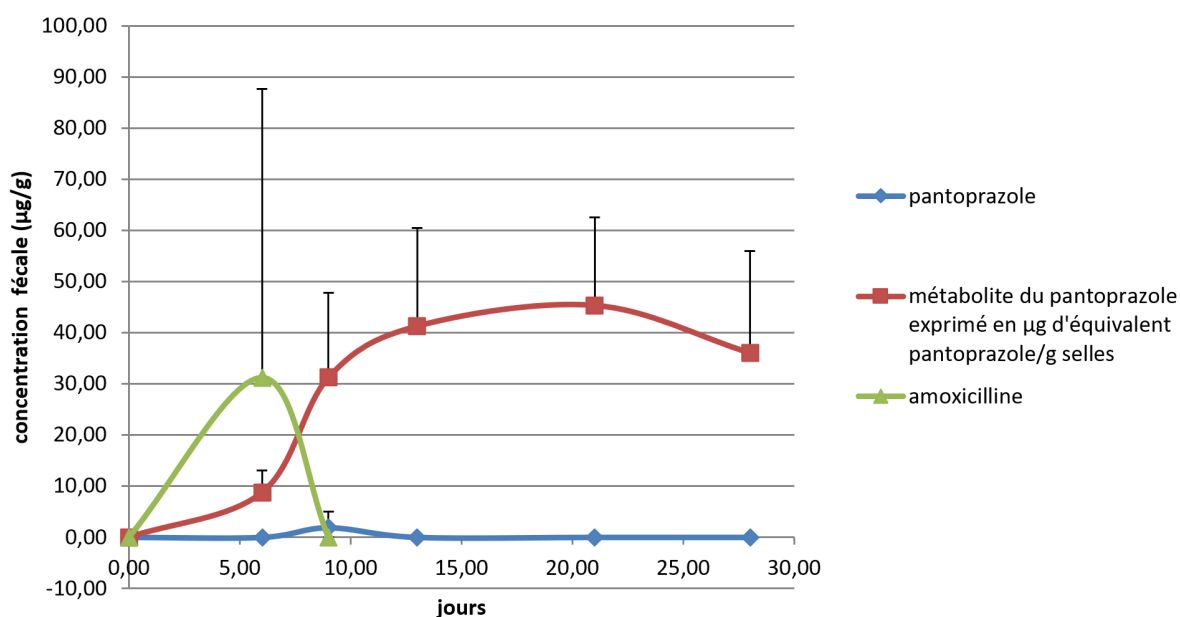
On n'observait pas de différences de concentration du titre fécal en *E. coli* BLSE entre les deux groupes (Figure 12 ;  $p > 0,05$  pour chaque point en analyse univariée ;  $p > 0,05$  RM 2-way ANOVA ;  $p > 0,05$  en comparant les AUC).



	Souris n°4			Souris n°5			Souris n°6		
	J8	J11	J18	J8	J11	J18	J8	J11	J18
PEC02	1,45x10 <sup>4</sup>	ND	ND	2,29x10 <sup>6</sup>	ND	ND	ND	8,51x10 <sup>4</sup>	ND
PEC08	ND	ND	ND	2,87x10 <sup>5</sup>	ND	ND	ND	ND	ND
PEC16	ND	ND	ND	ND	ND	ND	ND	ND	ND
PEC18	4,71x10 <sup>1</sup>	ND	ND	2,44x10 <sup>4</sup>	ND	ND	ND	ND	ND

A j5 la concentration moyenne d'amoxicilline dans les selles était de  $31,2 \pm 56,4$   $\mu\text{g/g}$  de selles. Au lendemain de l'arrêt de l'amoxicilline on ne retrouvait plus de concentrations détectables dans les selles (Figure 13).

Le pantoprazole, dans sa forme inchangée, a été détecté seulement à j8 avec une concentration moyenne de  $1,9 \pm 3,1$   $\mu\text{g/g}$  de selles. En revanche la concentration fécale d'un métabolite du pantoprazole a augmenté progressivement pour atteindre un maximum à j21 avec une moyenne de  $45,4 \pm 17,2$   $\mu\text{g}$  d'équivalent pantoprazole/g de selles (Figure 13).

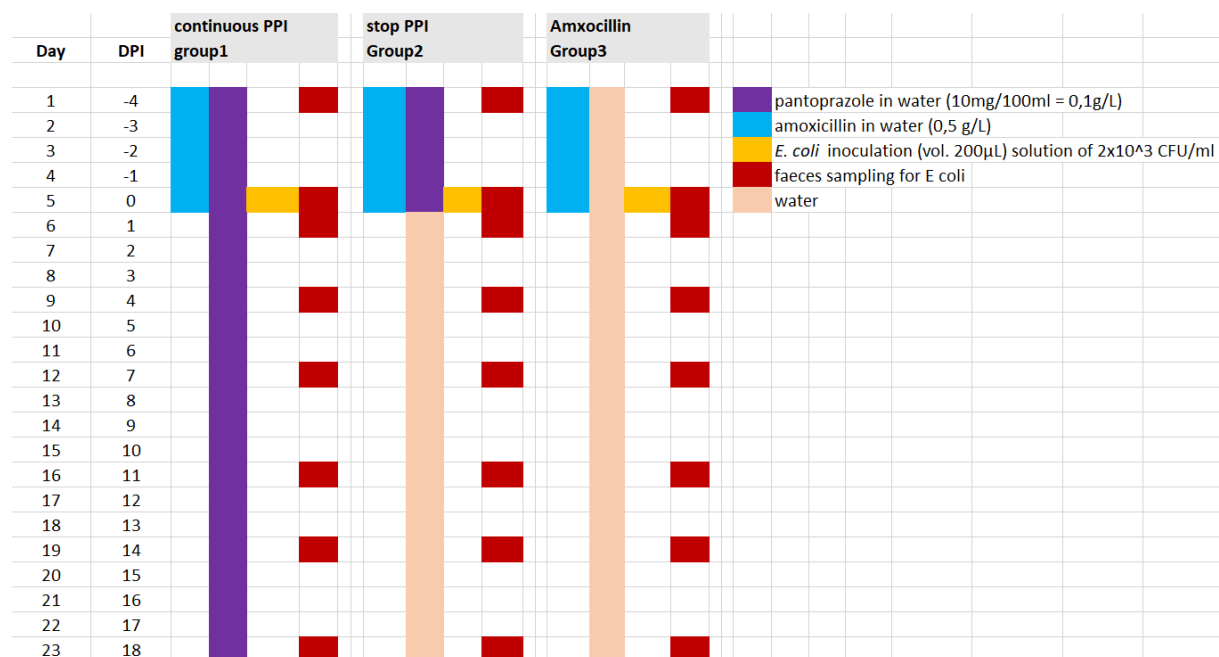


**Figure 13.** Concentration fécale en médicaments.

## 2.4. Effets des IPP sur la colonisation digestive en *E. coli* BLSE

### 2.4.1. Méthodes

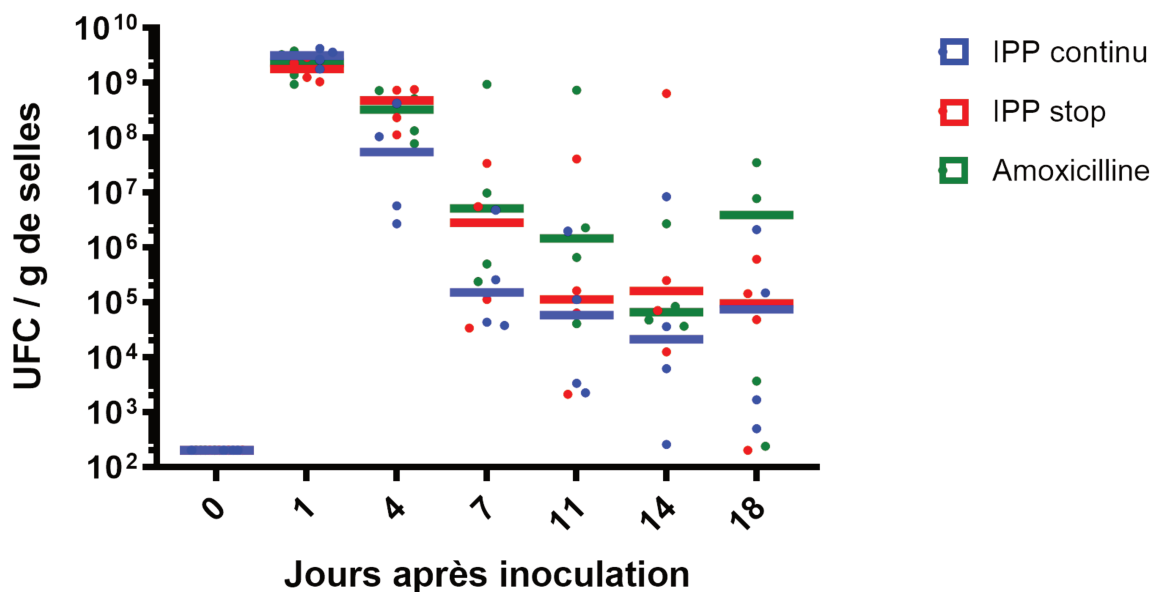
Douze souris SWISS mâle de 6 semaines de vie ont été réparties en trois groupes. Elles étaient exposées à trois schémas thérapeutiques différents. Le premier groupe a reçu de l'amoxicilline dans l'eau de boisson (0,5 g/L) pendant les cinq premiers jours et du pantoprazole dans l'eau de boisson (0,1 g/L) durant toute la durée de l'expérience ; le deuxième groupe a été exposé à un traitement oral d'amoxicilline et de pantoprazole, aux mêmes concentrations que le premier groupe, pendant les cinq premiers jours ; le dernier groupe n'a reçu que de l'amoxicilline (0,5 g/L) durant les cinq premiers jours (Figure X). Les biberons étaient renouvelés tous les trois jours. Au 5<sup>ème</sup> jour les souris recevaient l'inoculum bactérien par *E. coli* BLSE par gavage gastrique d'une solution de 200 µL correspondant à un inoculum de  $2 \times 10^3$  UFC. Les selles ont été récoltées et mises en culture (cf « Materials and methods » de l'article ci-dessus) à j-4, j0, j4, j7, j6, j11, j14 et j18 post inoculation (Figure 14). (APAFIS : 27847, Référence du dossier : 2020102017547615)



**Figure 14.** Schéma expérimental.

### 2.4.2. Résultats

L'on n'observait pas de différences de concentration du titre fécal en *E. coli* BLSE entre les trois groupes (Figure 15 ;  $p > 0,05$  pour chaque point en analyse univariée ;  $p > 0,05$  RM 2-way ANOVA ;  $p > 0,05$  en comparant les AUC ; Figure 15).



**Figure 15.** Médianes (lignes) et données individuelles (points) des concentrations fécales en *E. coli* BLSE en fonction du groupe de traitement.

# Discussion

## 1. Obstacles à l'utilisation des phages au sein du tractus digestif

Notre revue de la littérature a soulevé de nombreux obstacles quant à l'utilisation des bactériophages pour une décolonisation digestive et notamment d'un portage asymptomatique (46). En effet, tout comme dans les modèles pulmonaires (49), une synergie avec le système immunitaire semble un facteur clef d'efficacité clinique. Le manque de preuves provenant de modèles expérimentaux et soutenant que l'application de phages par voie orale peut réduire efficacement les bactéries intestinales, peut être en partie lié à l'absence, dans ces modèles, d'un contexte de maladie qui pourrait fournir un environnement plus favorable à l'activité des phages. En effet, deux tiers des études incluses dans notre revue ont montré que les traitements par phages avaient une efficacité transitoire ou inexistante pour réduire les niveaux des bactéries cibles, montrant clairement que la phagothérapie intestinale n'est pas aussi facile qu'on pourrait s'y attendre. Trois principaux obstacles à l'efficacité des phages dans l'intestin ont fait l'objet de discussions.

Premièrement, les phages doivent atteindre l'intestin, deuxièmement, ils doivent trouver une cible sensible et troisièmement, ils doivent réussir à diminuer la concentration en bactéries cibles. La façon dont les phages surmonteront mécaniquement ces barrières dépendra non seulement de leur nature (défis physiques, chimiques ou biologiques) mais aussi de l'environnement auquel les phages seront confrontés aux trois échelles que sont l'hôte, l'organe et la cellule bactérienne. Outre le pH acide, les enzymes hydrolytiques peuvent également réduire le nombre de particules de phage transitant dans l'intestin. Une solution à la dégradation des particules de phage au cours du transit intestinal peut résider dans l'utilisation de phages encapsulés (50,51).

Une fois que les phages ont atteint la section intestinale où résident les bactéries cibles, ils doivent trouver un hôte sensible. En effet, l'environnement intestinal (comme la disponibilité des nutriments ou la concentration en oxygène, entre autres) affecte la physiologie bactérienne, qui à son tour pourrait affecter la sensibilité des bactéries aux phages (52–54). De plus, un agent pathogène perturbera probablement la physiologie de l'hôte, ce qui pourrait à son tour affecter la sensibilité bactérienne aux phages (55,56). Enfin, la distribution spatiale des phages et des bactéries dans les sections intestinales peut être bénéfique pour l'une ou l'autre des populations, favorisant leur coexistence, comme montré *in vivo* (58). La propriété de certains phages à se lier aux mucines altérerait leur diffusion, mais pourrait augmenter leur capacité à atteindre les bactéries incrustées dans cette structure extracellulaire située à la surface des cellules épithéliales (58,59). Par conséquent, les solutions pour surmonter les défis susmentionnés résident dans la sélection de phages avec des capacités améliorées pour atteindre leur cible, ce qui nécessiterait une connaissance préexistante sur le(s) comportement(s) spécifique(s) de ces cibles dans l'intestin.

Une fois qu'ils ont atteint la surface des cellules bactériennes, les phages doivent vaincre les systèmes de défense bactériens qui entraînent la croissance des bactéries résistantes aux phages. Ces défenses comprennent des mutations, des modifications de surface, des modifications de restriction, des infections abortives et des systèmes CRISPR (répétitions palindromiques courtes régulièrement espacées en cluster). A travers les âges de coévolution avec les bactéries, les phages ont vaincu les systèmes de défense en faisant évoluer des variants génétiques, en modifiant leurs bases nucléotidiques ou encore en inventant de nouvelles fonctions telles que les protéines anti-CRISPR. Réciproquement pour les bactéries, tous les phages ne possèdent pas la capacité de développer une grande variété de mesures de contre-résistance. Il a été également montré que les phages peuvent passer des bactéries sensibles aux bactéries résistantes dans l'intestin de souris par une seule mutation sur les fibres de leur queue, ce qui démontre que les phages peuvent

s'adapter assez rapidement aux populations bactériennes fluctuantes afin de persister dans cet organe (60). Chaque couple phage/bactérie possède son propre potentiel de coévolution, ce qui complique fortement le choix des phages et de leur combinaison (cocktail de phages) pour des applications thérapeutiques.

Les données les plus convaincantes, sur la phagothérapie intestinale, examinées dans notre revue de la littérature provenaient de modèles dans lesquels une infection intestinale symptomatique était en cours contrairement aux modèles de colonisation digestive asymptomatique. Les recommandations ESCMID-EUCIC concernant les stratégies de décolonisation digestive des sujets porteurs de bactéries Gram-négatives multirésistantes proposent d'évaluer urgemment l'efficacité des bactériophages dans cette indication par des essais contrôlés randomisés (61). Toutefois nous estimons que de nombreuses connaissances sont encore à acquérir et qu'une harmonisation de la recherche fondamentale est nécessaires avant d'initier de nouveaux essais randomisés sur l'homme (46). En effet le seul essai sur des enfants hospitalisés au Bangladesh pour diarrhées bactériennes (*E. coli* entérotoxigène essentiellement) a été un échec (62).

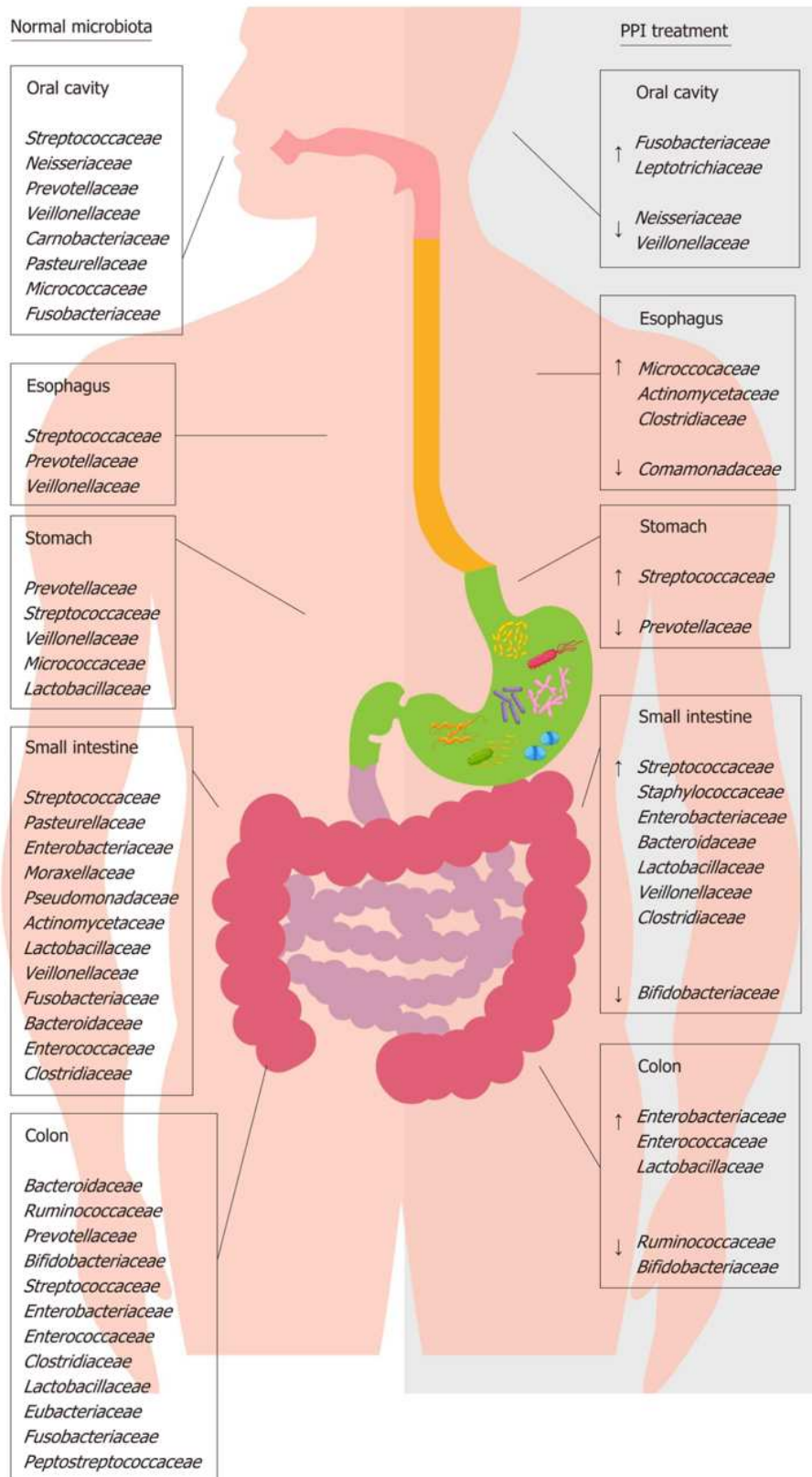
## 2. Modèles murins de colonisation digestive

Nous avons réussi à développer plusieurs modèles de portage intestinal d'*E. coli* BLSE grâce à l'administration de médicaments dans l'eau de boisson. L'avantage de nos modèles était que la bêta-lactamine et l'IPP étaient dilués dans l'eau des biberons, ce qui est plus simple que certains modèles précédents à injections sous-cutanées.(63–65)



### 3. Effets des inhibiteurs de la pompe à protons

Les inhibiteurs de la pompe à protons (IPP) font partie des dix médicaments les plus utilisés dans le monde. L'utilisation des IPP affecte le microbiome intestinal dans tout le tractus gastro-intestinal.(66) Imhann *et al.* ont observé une diminution significative de la diversité de Shannon et des changements dans 20% des taxons bactériens chez les utilisateurs d'IPP. Plusieurs bactéries orales étaient surreprésentées dans le microbiome fécal des utilisateurs d'IPP, notamment le genre *Rothia*, et une augmentation significative des bactéries : genres *Enterococcus*, *Streptococcus*, *Staphylococcus* et l'espèce potentiellement pathogène *Escherichia coli* (Figure 16).(67) L'utilisation des IPP a également été associée à un risque accru d'infections entériques, plus particulièrement de *Clostridium difficile*.(68) Mais un modèle murin récent a montré que l'oméprazole ne favorise pas la colonisation de *Clostridioides difficile*, et a un impact minime sur la structure du microbiote murin.(69)



**Figure 16.** Distribution des principales familles bactériennes du microbiote humain dans des conditions physiologiques et pendant le traitement par inhibiteurs de la pompe à protons (d'après Bruno *et al.* 2019 (66)).

Une méta-analyse récente, comprenant 26 études d'observation, a révélé que l'utilisation de supprimeurs d'acide était associée à un risque accru de colonisation du tractus intestinal par des micro-organismes multirésistants de l'ordre des *Enterobacterales* (produisant des  $\beta$ -lactamases à spectre étendu, des carbapénémases ou des  $\beta$ -lactamases AmpC à médiation plasmidique) et par des entérocoques résistants à la vancomycine. Cette association est biologiquement plausible mais doit être interprétée avec prudence, car les preuves issues d'études observationnelles ne peuvent pas prouver la causalité.(70)

Dans un modèle murin, Stiefel *et al.* ont montré que la suppression de la production d'acide gastrique par un traitement par IPP facilite la colonisation du gros intestin par des *Enterococcus spp.* résistants à la vancomycine (ERV) et *Klebsiella pneumoniae* chez des souris traitées à la clindamycine. En bref, pour les expérimentations réalisées par Stiefel *et al.*, les souris ont reçu du pantoprazole (0,4 mg dans 0,2 ml) ou une solution saline normale par voie sous-cutanée deux fois par jour pendant deux jours. Une dose de clindamycine sous-cutanée (4 mg) a été administrée 2 h avant la provocation bactérienne (100 UFC dans 50  $\mu$ l de solution saline normale). Le traitement par IPP a facilité l'établissement de la colonisation du gros intestin par les ERV (75 à 80 %, contre 20 à 25 % pour les témoins traités par une solution saline) et par *K. pneumoniae* à BLSE (90 %, contre 30 % pour les témoins traités par une solution saline) sur une période d'étude de 3 jours.(63) Durant nos expériences, nous avons montré que, chez les souris C57BL/6J, l'ajout d'un IPP à l'antibiothérapie non seulement augmentait la concentration fécale initiale de BLSE, mais qu'elle persistait également à des titres plus élevés, loin du défi bactérien. Cet effet facilitateur de la persistance de la colonisation dans notre modèle correspond bien aux observations sur l'homme.(70) Le microbiome fécal des utilisateurs d'IPP est altéré avec une surreprésentation des bactéries orales.(67,71) Les altérations du microbiome intestinal induites par les IPP

pourraient donc favoriser une colonisation digestive prolongée par des MDRO, mais des travaux supplémentaires sont nécessaires pour étayer cette hypothèse. En effet, les effets des IPP sur la colonisation digestive d'E. coli BLSE semblent moins prononcés chez les souris SWISS que C57BL/6J (données du laboratoire non publiées). Cette différence pourrait être expliquée par une composition différente du microbiote intestinale entre ces deux espèces.

#### 4. Modèles de décolonisation de bactéries multi-résistantes grâce aux phages

Dans notre modèle avec une pression continue d'antibiothérapie, nous n'avons pas observé d'efficacité durable de la phagothérapie. Ces résultats sont en accord avec les expériences de Galtier *et al.* qui, dans leur modèle à pression continue d'antibiothérapie, n'ont trouvé aucune efficacité. En revanche, une dose unique d'un cocktail de trois phages a eu une bonne efficacité sur le portage digestif d'E. coli uropathogènes lorsque la pression antibiotique n'était plus présente.(72) Les inhibiteurs de la pompe à protons entraînent également des modifications du microbiote intestinal, ce qui peut avoir influencé nos résultats puisque nous n'avons pas eu affaire à une pression antibiotique continue mais à une pression continue par les IPP.

Pour que les phages puissent infecter avec succès les bactéries cibles dans l'intestin, ils doivent pouvoir les atteindre et entrer en contact avec elles.(46) Le problème identifié est la dégradation des phages pendant le transit intestinal et, surtout, pendant le passage gastrique à faible pH.(73–75) Pour contrer ce phénomène, nous avons utilisé des IPP pour augmenter le pH gastrique, en plus de la micro-encapsulation des phages. L'encapsulation limite efficacement la réduction de la concentration des phages dans le tractus gastro-intestinal après administration orale.(76) En effet, les phages ne sont généralement pas stables dans un environnement aussi acide que l'estomac et certaines études utilisent

empiriquement un tampon de bicarbonate lors de l'administration orale des phages.(74,76) Cependant, la seule étude sur *E. coli* (O157:H7 résistant à l'acide nalidixique) que nous avons trouvée dans la littérature utilisant des phages encapsulés n'a pas montré d'efficacité in-vivo dans un modèle bovin.(77) En revanche, *E. coli* semble être capable de survivre pendant plusieurs jours dans un environnement acide.(78) D'autres modèles animaux ont été développés avec des phages encapsulés pour traiter les infections à Salmonella, et les résultats sont encourageants.(79,80) Cependant, ces modèles d'infection sont quelque peu différents des modèles de colonisation digestive asymptomatique.(46)

## 5. Etat des lieux et perspectives de la phagothérapie chez l'humain

Il existe actuellement peu de preuves solides concernant l'efficacité de la phagothérapie en médecine humaine. Nous proposons un état des lieux des études randomisées publiées à ce jour.

### 5.1. Tractus digestif

L'étude de Sarker et al. publiée en 2016 concernant les enfants souffrant de diarrhée bactérienne, principalement à *E. coli* entérotoxigène, n'a pas montré d'efficacité clinique ni microbiologique de deux cocktails de bactériophages administrés par voie orale (T4-like phage cocktail et Microgen ColiProteus phage cocktail). Lors de l'analyse intermédiaire l'étude a été arrêtée prématurément, après l'inclusion de 120 sujets, pour inefficacité.(62)

En utilisant un cocktail de quatre phages ciblant *E. coli*, l'étude PHAGE (2019) a montré qu'un traitement quotidien de 28 jours diminuait significativement chez l'adulte sain. Néanmoins seuls 21 sujets sur 43 (49%) avaient un niveau détectable d'*E. coli* avant le traitement (phages ou placebo). Parmi eux le taux de réponse était de 71% avec les phages (réduction moyenne en *E. coli* d'environ 40%) et de 47%

avec le placebo (réduction moyenne de 14%). En revanche il n'y avait pas d'effet concernant l'alpha et la bêta diversité du microbiote digestif ; et d'autres espèces étaient affectées par les phages comme *Eubacterium* et *Clostridium perfringens*.(81) L'étude PHAGE-2, publiée en 2020, a étudié l'ajout d'un cocktail de phage (PeforPro) à un probiotique (*B. lactis*) sur 68 participants et n'a pas retrouvé d'effet clinique probant à partir d'un questionnaire évaluant l'état de santé gastro-intestinale. En revanche le microbiote des sujets dans le groupe phage retrouvait une augmentation en *Lactobacillus* et en taxons microbiens producteurs d'acides gras à chaîne courte.(82)

### 5.2. Peau

Une étude de phase 1, publiée en 2009, a évalué la sécurité de l'application de bactériophages ciblant *Pseudomonas aeruginosa*, *Staphylococcus aureus* et *Escherichia coli* pendant 12 semaines sur chez des sujets souffrant d'ulcère veineux chronique. L'étude a conclu qu'il n'y avait pas plus d'effets indésirables chez les patients traités que dans le groupe contrôle, ni d'ailleurs d'impact sur l'amélioration clinique.(83)

L'étude PhagoBurn (2019) a tenté d'évaluer l'efficacité et la tolérance et de l'application locale d'un cocktail de 12 phages ciblant *Pseudomonas aeruginosa* chez des sujets brûlés et infectés par cette bactérie par rapport aux soins standards (crème de sulfadiazine d'argent 1 %). L'étude a été arrêtée prématurément après l'analyse de 25 sujets en raison du manque d'efficacité de la phagothérapie. En effet le délai médian de réduction durable de la charge bactérienne était de 144 heures dans le groupe traité par phages contre 47 heures dans le groupe standard.(84)

### 5.3. Tractus urinaire

Une étude géorgienne publiée en 2021 évaluait l'efficacité de l'administration intravésicale de bactériophages pour le traitement des infections des voies urinaires chez les patients subissant une résection transurétrale de la prostate. Le

cocktail de phages (Pyophage) était actif sur *Enterococcus* spp, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus* spp, et *Streptococcus* spp). Les sujets inclus avaient une infection des urines à l'une de ces bactéries sans présenter de signes généraux (absence de fièvre notamment). Cent-treize sujets ont été randomisés en trois groupes : placebo (irrigation vésicale), antibiothérapie, phagothérapie. La phagothérapie n'était pas supérieure au placebo que ce soit en terme clinique ou microbiologique.(85)

### 5.3. ORL

En 2009 une équipe anglaise a publié des résultats préliminaires encourageants d'une étude menée sur des sujets atteints d'otite chronique à *Pseudomonas aeruginosa* résistant aux antibiotiques. Vingt-quatre patients ont été inclus et 12 d'entre eux traités par bactériophages. Il en a résulté une discrète et transitoire amélioration clinique mais pas au niveau bactériologique par rapport au placebo.(86)

Une étude russe, publiée en 2021, a inclus 40 patients atteints de rhinosinusite chronique avec polypes nasaux. Après une chirurgie programmée des polypes, les patients s'appliquaient un gel intranasal 2 fois par jour pendant 10 semaines contenant soit un placebo soit un cocktail de 32 phages (Otofag) actifs sur de nombreuses espèces bactériennes pathogènes. Le nombre total de bactéries présentes dans les cavités nasales a diminué tandis que seules deux bactéries cibles ont vu leur concentration nasale diminuée (*Streptococci* et *Enterobacteriaceae*). Des résultats mitigés et transitoires concernant des marqueurs de l'inflammation ont également été reportés dans l'article.(87)

### 5.4. Etudes en cours ou à venir et perspectives

Dix-sept essais randomisés sont recensés en mars 2022 sur la plateforme Clinical Trials (Tableau 13). Ils concernent principalement le pied diabétique, la mucoviscidose, les infections urinaires et les infections des prothèses articulaires.

**Tableau 13.** Principaux essais randomisés en cours ou à venir concernant la phagothérapie, inscrits sur ClinicalTrials.gov.

NCT : National Clinical Trial

<b>NCT number</b>	<b>Title</b>	<b>Status</b>	<b>Participants</b>	<b>Conditions</b>
NCT04787250	Bacteriophage Therapy in Patients With Prosthetic Joint Infections	Not yet recruiting	78	Prosthetic Joint Infection
NCT05269134	Bacteriophage Therapy in Patients With Prosthetic Joint Infections Who Previously Failed Surgery for PJI (ACTIVE2)	Not yet recruiting	280	Prosthetic Joint Infection
NCT05177107	Bacteriophage Therapy in Patients With Diabetic Foot Osteomyelitis	Recruiting	75	Diabetic Foot Osteomyelitis
NCT04803708	Bacteriophage Therapy TP-102 in Diabetic Foot Ulcers (REVERSE)	Recruiting	26	Diabetic Foot Ulcer
NCT02664740	Standard Treatment Associated With Phage Therapy Versus Placebo for Diabetic Foot Ulcers	Not yet recruiting	60	Diabetic Foot Staphylococcal Infections



	Infected by S. Aureus (PhagoPied)			
NCT04815798	Phage Therapy for the Prevention and Treatment of Pressure Ulcers.	Not yet recruiting	69	Pressure Ulcer
NCT05240300	A Study of Topical BX005-A in Subjects With Moderate to Severe Atopic Dermatitis	Not yet recruiting	48	Atopic Dermatitis
NCT04191148	Safety, Tolerability, and PK of LBP-EC01 in Patients With Lower Urinary Tract Colonization Caused by E. Coli	Completed	36	Urinary Tract Infections
NCT04287478	Bacteriophage Therapy in Patients With Urinary Tract Infections	Recruiting	156	Urinary Tract Infection Bacterial
NCT05182749	Safety and Efficacy of the Bacteriophage Preparation, ShigActive™, in a Human Experimental Model of Shigellosis	Not yet recruiting	52	Shigellosis

NCT03808103	Safety and Efficacy of EcoActive on Intestinal Adherent Invasive E. Coli in Patients With Inactive Crohn's Disease	Recruiting	30	Crohn Disease
NCT04325685	The Effect of Supraglottic and Oropharyngeal Decontamination on the Incidence of Ventilator-associated Pneumonia (SGDC-VAP)	Unknown	60	Prevention of ventilator - associated pneumoniae
NCT04684641	CYstic Fibrosis bacterioPHage Study at Yale (CYPHY)	Recruiting	36	Cystic Fibrosis
NCT04596319	Ph 1/2 Study Evaluating Safety and Tolerability of Inhaled AP-PA02 in Subjects With Chronic Pseudomonas Aeruginosa Lung Infections and	Recruiting	48	Cystic Fibrosis

	Cystic Fibrosis (SWARM-Pa)			
NCT05010577	Nebulized Bacteriophage Therapy in Cystic Fibrosis Patients With Chronic Pseudomonas Aeruginosa Pulmonary Infection	Not yet recruiting	32	Cystic Fibrosis
NCT05184764	Study Evaluating Safety, Tolerability, and Efficacy of Intravenous AP-SA02 in Subjects With S. Aureus Bacteremia (diSArm)	Not yet recruiting	50	Staphylococcus Aureus Bacteremia
NCT04935892	Slippers to Reduce Transfer From Floors to Surfaces	Completed	34	Transmission

Malgré le fait que les preuves actuelles de l'efficacité des bactériophages en médecine reposent quasi exclusivement sur des case reports, de nombreux auteurs semblent très (trop ?) enthousiastes concernant leur utilisation en pratique courante.(88,89) D'autres études randomisées de qualité et de grande ampleur sont donc nécessaires.(89) Les phages sont certes considérés comme une thérapeutique ne présentant pas de danger particulier mais de nombreuses connaissances restent à acquérir, notamment sur les modèles animaux, avant d'envisager des études cliniques

de grande ampleur et particulièrement dans des milieux complexes comme le tractus digestif.(46) Pour ces raisons nous n'avons pas à ce jour entrepris la rédaction d'un protocole de recherche chez l'humain afin d'évaluer la capacité de la phagothérapie à éliminer un portage digestif de bactéries multirésistantes. De plus certains industriels, comme la société Clean Cells qui a financé les travaux de recherche de cette thèse, ont décidé d'arrêter leurs recherches et productions de bactériophages.

## Conclusions

Un traitement oral à l'amoxicilline a permis de favoriser le portage digestif chez la souris, ce qui était également le cas pour l'ajout d'IPP. Cependant, notre étude confirme la difficulté d'atteindre l'efficacité de la phagothérapie intestinale pour réduire le portage des bactéries multirésistantes *in vivo*. Seul un petit effet transitoire a pu être observé dans une expérience, tandis que les autres conditions expérimentales n'ont montré aucune efficacité.

Au total, le manque de succès de l'utilisation des phages dans les interventions thérapeutiques dans l'intestin reflète davantage notre connaissance limitée de cet écosystème que la capacité intrinsèque des phages à infecter les bactéries intestinales. En particulier, l'appréciation mécaniste des facteurs qui régissent l'activité des phages dans l'intestin est très faible. Les multiples modèles expérimentaux montrent clairement l'étendue des possibilités plutôt que de simplement mettre en évidence un chemin vers le succès. Afin d'améliorer nos connaissances obtenues à partir de modèles animaux, nous recommandons que les études futures intègrent les éléments suivants : (i) quantification en temps utile des particules de phages viables favorisant l'amplification des phages ; (ii) surveillance de la résistance aux phages au fil du temps et des traitements ; (iii) évitement des modèles pour lesquels le temps de transit bactérien est inférieur à 72 h, sauf s'ils reflètent des infections intestinales aiguës ; et (iv) administration des phages au moins 4 h après la provocation bactérienne pour permettre aux bactéries de s'adapter à l'environnement intestinal, sauf si un prétraitement (au moins 4 h avant la provocation bactérienne) est évalué. Les recherches futures dans ce domaine devront faire le lien entre les données issues des études métagénomiques et les modèles expérimentaux simplifiés. Une façon de progresser dans cette direction serait de construire des systèmes synthétiques de plus en plus complexes, tels que

ceux basés sur des modèles murins gnotobiotiques/isobiotiques.(90) Bien qu'encore imparfaits à plusieurs égards, ces modèles offrent une base de reproductibilité très élevée qui permettrait de répondre à des questions spécifiques concernant les mécanismes moléculaires. Néanmoins, les données les plus convaincantes sur la phagothérapie intestinale provenaient de modèles dans lesquels une maladie intestinale était en cours, ce qui correspond directement à la liste croissante de traitements compassionnels réussis actuellement et fournis aux patients confrontés à l'inefficacité thérapeutique des traitements antibiotiques. Cela plaide en faveur de la nécessité de prendre en compte l'hôte, en plus des bactéries et des phages, dans l'identification des conditions requises pour une efficacité optimale des applications de phages dans l'intestin.

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## Annexes

### AVIS DU COMITÉ D'ÉTHIQUE

(à transmettre au secrétariat autorisation de projet :  
[autorisation-projet@recherche.gouv.fr](mailto:autorisation-projet@recherche.gouv.fr))

Date : 18 décembre 2017

Référence du dossier : 201708291549991 / APAFIS 11056

Titre du Projet : Evaluation de l'efficacité de l'éradication  
 d'Entérobactéries productrices de BLSE du tractus digestif murin  
 par phagothérapie

1- Avis éthique sur le projet :  Favorable  
 Défavorable

Motif(s) en cas d'avis défavorable :

2- Proposition de reclassement des procédures expérimentales selon le degré  
 de gravité indiqué par le responsable de projet :  oui  non

Procédure(s) reclassée(s) et proposition de reclassement :

- procédure n° : 2 et 4
  - reclassement : légère
- (le bloc Procédure(s) reclassée(s) et proposition de reclassement est à reproduire le cas échéant)

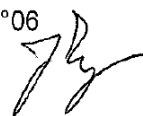
3- En application de l'article R.214-120 du décret n°2013-118 du 1<sup>er</sup> février 2013,  
 le projet devra t'il bénéficier d'une appréciation rétrospective à l'issue de sa  
 réalisation :  oui  non

4- Le cas échéant (cf point 4.3 du dossier), avis sur la réutilisation d'un animal  
 pour autant que l'animal n'ait pas été utilisé, préalablement à ce projet, plus  
 d'une fois dans une procédure expérimentale entraînant une douleur intense,  
 de l'angoisse ou une souffrance équivalente (article R.214-113 du décret  
 n°2013-118 du 1<sup>er</sup> février 2013) :

Favorable  Défavorable

Motif(s) en cas d'avis défavorable :

CEEA-PdL n°06  
 Dr Joël Eyer



### AVIS DU COMITÉ D'ÉTHIQUE

(à transmettre au secrétariat autorisation de projet :  
[autorisation-projet@recherche.gouv.fr](mailto:autorisation-projet@recherche.gouv.fr))

Date : 2 décembre 2020

Référence du dossier : 2020102017547615 / APAFIS 27847v4  
 Titre du Projet : Évaluation de l'impact d'un traitement par inhibiteur  
 de la pompe à proton (IPP) sur le portage digestif de bactéries  
 multi-résistantes aux antibiotiques dans un modèle murin.

1- Avis éthique sur le projet :  Favorable  
 Défavorable

Motif(s) en cas d'avis défavorable :

2- Proposition de reclassement des procédures expérimentales selon le degré  
 de gravité indiqué par le responsable de projet :  oui  non

Procédure(s) reclassée(s) et proposition de reclassement :

- procédure n° :
  - reclassement :
- (le bloc Procédure(s) reclassée(s) et proposition de reclassement est à reproduire le cas échéant)

3- En application de l'article R.214-120 du décret n°2013-118 du 1<sup>er</sup> février 2013,  
 le projet devra t'il bénéficier d'une appréciation rétrospective à l'issue de sa  
 réalisation :  oui  non

4- Le cas échéant (cf point 4.3 du dossier), avis sur la réutilisation d'un animal  
 pour autant que l'animal n'ait pas été utilisé, préalablement à ce projet, plus  
 d'une fois dans une procédure expérimentale entraînant une douleur intense,  
 de l'angoisse ou une souffrance équivalente (article R.214-113 du décret  
 n°2013-118 du 1<sup>er</sup> février 2013) :

Favorable  Défavorable

Motif(s) en cas d'avis défavorable :

CEEA-PdL n°06  
 Pr Jean-Claude Desfontis



## Abstracts 2020



## Abstract 571

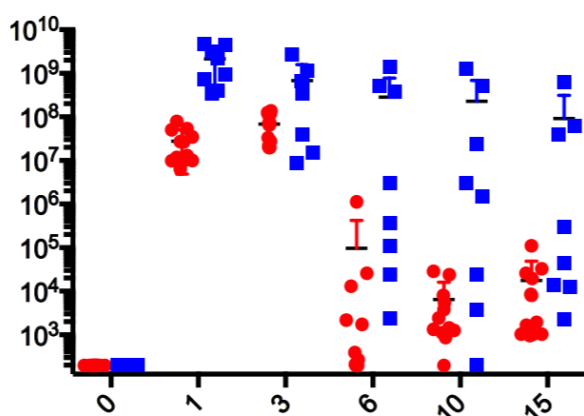
**Proton pump inhibitors increase the digestive carrying of OXA-48-producing *Enterobacteriaceae* in a mouse model**
François Javaudin<sup>\*1</sup>, Quentin Le Bastard<sup>1</sup>, Michel Dion<sup>1</sup>, Yihienew Bezabih<sup>1</sup>, Emmanuel Montassier<sup>1</sup>, Eric Batard<sup>1</sup><sup>1</sup>University of Nantes, MiHAR, Nantes, France

**Background:** Proton pump inhibitors (PPI) are responsible for altering the composition of the gut microbiota but little is known about their impact on the gut resistome. It would appear that PPI consumption is associated with extended-spectrum  $\beta$ -lactamase-producing *Enterobacteriaceae* rectal carriage at hospital admission. Our objective was to evaluate the effect of PPI on the digestive carriage of OXA-48-producing *Enterobacteriaceae* in a mouse model.

**Materials/methods:** C57BL/6J mice were initially treated orally with amoxicillin (0.5 g.l<sup>-1</sup> in drinking water) for 7 days before the bacterial challenge (10<sup>7</sup> CFU of OXA-48-producing *Escherichia coli*) by gastric gavage. PPI were added to drinking water (0.1 g.l<sup>-1</sup> of pantoprazole) for a group of mice (PPI group) throughout the experiment while the control group did not receive them. The stool was collected for 15 days after the bacterial challenge. The bacterial count was performed on selective chromogenic medium for the screening of OXA-48 type Carbapenemase-Producing *Enterobacteriaceae*. The mice were housed in individual cages to avoid inter-individual contamination.

**Results:** Height mice were analyzed in the PPI group and 12 in the control group. One day after the bacterial challenge the PPI group had a higher average concentration of *E. Coli* OXA-48 in the feces (2.1x10<sup>9</sup> CFU.g<sup>-1</sup> versus 2.8x10<sup>7</sup>; P < 0.001). Except for the 3rd day after the bacterial challenge, this concentration was higher in the PPI group than in the control group (P < 0.001 on days 6, 10 and 15). Indeed, there was a clear decrease in *E. Coli* OXA-48 colonization in the control group from the 6th day post-bacterial challenge (approximately 4 log CFU.g<sup>-1</sup>), while the PPI group kept a high level of carriage (approximately 8 log CFU.g<sup>-1</sup>) (Figure).

**Conclusions:** The use of PPI could be a factor promoting digestive colonization with resistant bacteria. These widely prescribed treatments should be evaluated in humans in order not to ignore a possible factor promoting the spread of this type of bacterium.



**Titre :** Évaluation de l'efficacité de l'éradication d'entérobactéries productrices de BLSE du tractus digestif murin et humain par phagothérapie

**Mots clés :** phagothérapie, bêtalactamases à spectre étendu, bactéries multirésistantes, colonisation digestive, *Escherichia coli*

**Résumé :** La résistance croissante des bactéries aux antibiotiques est un problème de santé publique mondial majeur. Le microbiote intestinal constitue un réservoir important de cette résistance. Cependant, peu de données sont disponibles sur la capacité de la phagothérapie à réduire le portage digestif de bactéries multirésistantes.

Quatre nouveaux phages ont été isolés in vitro pour leur efficacité contre une souche d'*E. coli* productrice de bêta-lactamase à spectre étendu (BLSE) associé à une carbapénémase OXA-48. Nous avons développés plusieurs modèles murins de colonisation digestive à *E. coli* BLSE associant l'administration d'amoxicilline (0,5 g/L) et/ou de pantoprazole (0,1 g/L) dans l'eau de boisson.

Quatre modèles de phagothérapie ont été testés contre placebo en mesurant la concentration fécale en *E. coli* BLSE. Pour un de ces modèles les phages étaient encapsulés. À part une légère diminution significative transitoire (-0.71 log CFU/g ;  $p=0,02$ ) dans un des modèles, il n'a pas été mis en évidence de différence en concentration fécale d'*E. coli* BLSE avec la phagothérapie par rapport au placebo.

L'efficacité des phages dans le tractus digestif est soumise à de nombreux obstacles : progression jusqu'à l'intestin, atteinte de leur cible et réussite de l'infection. D'autres connaissances sont encore nécessaires afin d'évaluer la capacité des phages à agir dans le tractus digestif et notamment en ce qui concerne les modèles de colonisation.

**Title:** Evaluation of the efficacy of eradication of ESBL-producing Enterobacteriaceae from the murine and human digestive tract by phagotherapy

**Keywords:** phage therapy; extended-spectrum beta-lactamase; multidrug-resistant bacteria; intestinal carriage; *Escherichia coli*

**Abstract:** The growing resistance of bacteria to antibiotics is a major global public health concern. An important reservoir of this resistance is the gut microbiota. However, limited data are available on the ability of phage therapy to reduce the digestive carriage of multidrug-resistant bacteria.

Four novel phages were isolated in vitro for efficacy against an extended-spectrum beta-lactamase-producing (ESBL) *E. coli* strain associated with a carbapenemase OXA-48. We have developed several murine models of ESBL *E. coli* colonisation combining the administration of amoxicillin (0.5 g.L<sup>-1</sup>) and/or pantoprazole (0.1 g.L<sup>-1</sup>) in drinking water.

Four phage therapy models were tested against placebo by measuring the faecal ESBL *E. coli* concentration. For one of these models the phages were encapsulated. Apart from a slight transient decrease (-0.71 log CFU.g<sup>-1</sup>;  $p=0.02$ ) in one of the models, there was no difference in faecal ESBL *E. coli* concentration with phage therapy compared to placebo.

The effectiveness of phages in the digestive tract is subject to many obstacles: progression to the intestine, reaching their target and successful infection. More knowledge is still needed to assess the ability of phages to act in the digestive tract, particularly with regard to colonisation models.