Phage therapy: from case-based translational research to Gut-On-A-Chip model of digestive decolonization

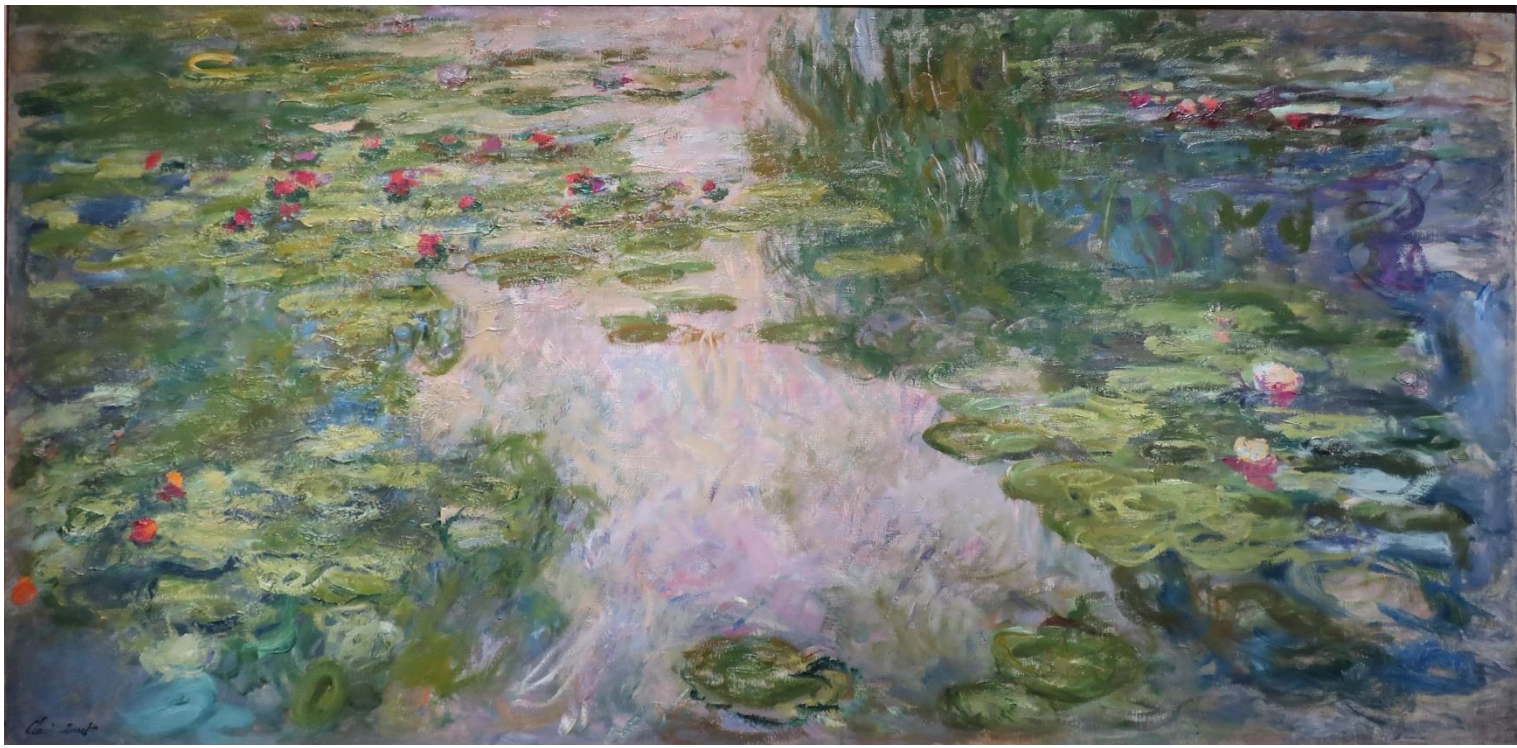
Brieuc Van Nieuwenhuyse

Juillet 2024

Thèse présentée en vue de l'obtention du grade

de docteur en sciences médicales

Secteur des sciences de la santé



Cover image :

Claude Monet – Le Bassin aux nymphéas

1917-1919, Giverny, France

Honolulu Museum of Art

Public Domain

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

34

35

36

Nous sommes le champ et le laboureur, le moissonneur et la moisson.

Khalil Gibran, Le précurseur, 1920

_____

If travel is searching
And home has been found
I'm not stopping
I'm going hunting

Björk, Hunter, 1997

55

56

57

58

59

60

61

62

63

64

65

66

67

68

69

70

71

72

FACULTÉ DE MÉDECINE

UCLOUVAIN

INSTITUT DE RECHERCHE EXPÉRIMENTALE ET CLINIQUE

PÔLE PÉDIATRIQUE

PHAGE THERAPY: FROM CASE-BASED TRANSLATIONAL RESEARCH TO GUT-ON-A-CHIP MODEL OF DIGESTIVE DECOLONIZATION

Brieuc VAN NIEUWENHUYSE

Thesis submitted in fulfillment of the requirements for the degree of "Docteur en Sciences Médicales"

Promotor: Professor Dimitri VAN DER LINDEN

Co-Promotor: Professor Leïla BELKHIR

2024

92

93

94

95

96

97

98

99

100

101

102

103

104

105

106

107

108

109

Remerciements

L'aventure doctorale touche désormais pour moi à sa fin, après plus de quatre ans d'un parcours qui aura connu de nombreux rebondissements dès sa genèse, et aura été riche d'innombrables apprentissages. L'occasion est donc toute désignée pour une (une seule ?) minute de remerciements. Cruauté du sort, l'écriture de ce passage d'importance pourtant capitale arrive, comme toujours, à un stade où l'envie ultime du doctorant est … d'arrêter d'écrire, enfin. Mais pas question de reculer devant l'effort. La thèse est un épisode professionnel et personnel unique dont la caractéristique la plus frappante est, à mon sens, d'être constituée de contrastes. Il est un de ces contrastes qui m'a particulièrement marqué : la solitude souvent extrême inhérente au parcours d'une part, et d'autre part, pourtant, la nécessité incontournable de collaborer, de savoir bien s'entourer, de frapper aux bonnes portes. C'est à ce besoin essentiel de contact, d'entraide et de partage que je veux rendre hommage ici.

Si je ne pouvais remercier que deux personnes, il s'agirait naturellement du **Professeur Dimitri Van der Linden**, mon promoteur, et du **Professeur Leïla Belkhir**, ma co-promotrice. Leur bienveillance, leur enthousiasme, leur rigueur, leur disponibilité, leurs nombreuses idées et suggestions ont été les garants de ma progression lors de ce parcours. Mais plus encore que le reste, j'aimerais particulièrement remercier et saluer leur confiance en mes propres projets et le libre-arbitre quasi-total qu'ils m'ont laissé : ils ont donné à cette thèse le parfum de l'auto-détermination et de la découverte de l'inconnu. Leur bonne entente de longue date et leur

complémentarité de tempéraments, de domaines d'expertise et d'idées ont également été des atouts de poids.

J'ai croisé pour la première fois le chemin du **Professeur Dimitri Van der Linden** il y a désormais dix ans : le simple fait de l'écrire me donne presque le vertige tant le temps est passé vite. Il était alors mon professeur de microbiologie en bachelier à l'Université de Namur. Notre parcours commun a débuté par un de ces concours de circonstances dont la vie a le secret : dans le décours d'un échange de mails sur une mystérieuse affaire de morsure de tique, de famille, d'allergie et de viande hachée, le Professeur Van der Linden m'a proposé, lorsque j'arriverais à Woluwé un peu plus tard, d'entamer sous sa supervision un mémoire de recherche clinique. Dix-huit mois plus tard, fin 2016 dans son bureau des Cliniques universitaires Saint-Luc, il me propose initialement deux sujets de recherche, l'un concernant les infections à *Borrelia*, l'autre le screening de la tuberculose en pays à faibles revenus. Ce à quoi il ajoute, peu après "ou alors … j'ai quelque chose de plus exotique". C'est ce jour-là que j'entendis parler de virus bactériophages pour la première fois ; voilà maintenant plus de sept ans qu'ils me passionnent. Ce seul point justifie tous les remerciements. Je lui en dois néanmoins bien d'autres pour sa gentillesse et son sourire connus de générations d'étudiants, sa disponibilité, son sens du rêve et de l'importance de ce qui se joue hors du travail, sa confiance en mes capacités, et ses conseils, bien sûr, toujours précieux.

C'est à la transition entre le mémoire de recherche clinique et la thèse que le **Professeur Leïla Belkhir** a accepté de devenir ma co-promotrice de thèse. Elle a dès lors été un soutien indéfectible à mon travail et à mes idées tout en sachant toujours conserver un œil critique sur ceux-ci, et faisant

preuve d'une forme de rigueur qui avait autant vocation à canaliser mon enthousiasme qu'à l'encourager. Elle a fait preuve d'une disponibilité sans faille dans certains moments de doute parfois vertigineux, libérant au pied levé un créneau dans sa journée déjà surchargée pour me recevoir (autour d'un café et d'un morceau de chocolat, on ne peut pas se tromper …), pour m'écouter d'abord, en toute empathie, puis me guider ensuite grâce à un sens pratique et efficace que tous lui connaissent … et qui me fait bien souvent défaut !

L'un et l'autre ont également toujours eu à cœur de m'impliquer dans leurs projets cliniques de sorte à me donner l'occasion, rare et précieuse pour un doctorant, de venir directement éprouver les fruits de mes recherches et questionnements au contact de la "vraie vie" de la pratique clinique. J'aimerais enfin saluer leur sens extrême du travail, voire même du sacrifice, dont ils ont particulièrement fait preuve à l'occasion de la crise du COVID-19. L'admiration que je leur témoigne n'en est que plus grande.

J'adresse également mes sincères remerciements à mon jury de thèse, qui a toujours su se montrer disponible et constructif au long de ces quatre années.

Le **Professeur Isabelle Leclercq**, qui le préside, m'a inspiré par sa franchise, son sens du détail, sa motivation à me lancer dans le nouveau, l'inexploré. Elle a su mettre à l'épreuve mes préconceptions et mes idées reçues, notamment en début de parcours, et stimuler ainsi mon imagination.

Le **Professeur Etienne Sokal**, qui préside le pôle pédiatrique de l'IREC qui m'a accueilli pendant quatre ans, a lui aussi eu l'occasion de me

mettre à l'épreuve durant nos nombreuses réunions de labo et a toujours cherché à nous tirer, les autres doctorants et moi-même, vers le haut en termes de rigueur scientifique et de vulgarisation orale. Exigeant, il n'a néanmoins jamais été avare de compliments lorsqu'il le jugeait justifié, et a toujours fait preuve d'une grande curiosité concernant mon sujet, pourtant fort éloigné de son expertise principale et des axes de recherche habituels du labo.

Le **Professeur Thomas Michiels** a également été une ressource précieuse, et l'initiateur de discussions pointues lors des comités d'accompagnement notamment, où son regard de fondamentaliste a apporté un éclairage nouveau sur mon travail et ses failles potentielles, toujours avec la plus grande bienveillance.

Le **Professeur Xavier Wittebole,** qui me suit depuis l'époque du mémoire, connait bien mon enthousiasme pour ce sujet qu'il connait lui-même en profondeur, et a su par conséquent faire preuve de rigueur et apporter la contradiction sur certains points, tout en étant toujours très encourageant.

Le **Docteur Jean-Paul Pirnay** fut une source d'inspiration et de connaissance inépuisable en tant qu'expert d'envergure internationale en phagothérapie : je tiens à le remercier pour sa gentillesse et son ouverture inconditionnelles, m'ayant en effet ouvert les portes du LabMCT en toute spontanéité, et m'ayant orienté vers de nombreux experts de sous-branches du sujet.

Je tiens enfin à remercier chaleureusement le **Professeur Pierre Smeesters** et le **Professeur Piet Cools** pour avoir accepté d'être les membres externes de mon jury final : leur curiosité, leur enthousiasme, leur expertise

respective et leur regard nouveau sur mon travail ont permis des discussions approfondies et des suggestions inédites durant la défense privée.

Je tiens ensuite, à remercier les membres du pôle PEDI, qui fut mon labo d'accueil. En tant qu'assistant en médecine interne, je n'étais pas spécialement destiné à évoluer dans un labo de pédiatrie. Les règles de l'administration ont néanmoins décidé pour moi, et je suis au final ravi qu'il en ait été ainsi ! Outre le Professeur Sokal déjà mentionné, qui le dirige, je me dois de remercier particulièrement, parmi les seniors, le **Professeur Xavier Stéphenne** et le **Professeur Isabelle Scheers**, qui ont été des contacts réguliers et complices de mes allers-retours au 92 pour le suivi de l'étude OPTICS en hépatologie pédiatrique, et qui ont toujours été source de bons conseils en réunions de labo. **Joachim Ravau** et **Jonathan Everaerts**, techniciens et véritables piliers de PEDI, m'ont régulièrement aidé dans mes manips, mes commandes, mes débouchages incessants de pompe d'aspi en chambre de culture, et bien plus encore. Mes co-doctorants furent autant de superbes rencontres. Dès les premiers jours, les "conseils de grand frère" de **Tanguy Demaret** ont catalysé mon intégration et ma prise en main de pas mal d'outils informatiques, notamment. Les nombreuses discussions avec **Giulia Jannone** ont toujours fait un bien fou, en alternance avec nos crises conjointes de "sugar craving" qui auraient probablement inquiété nos collègues chercheurs de diabéto. En parlant d'eux justement, **Olivier Pollé** fut également un comparse irremplaçable, toujours prêt à rigoler, boire un verre, ou résoudre tout problème imaginable par une bonne ligne de code. **Sophie Welsch** me faisait rêver au retour de ses conférences de crossfit en Floride, et

a été un modèle pour sa détermination dans ses derniers mois de thèse. Ceux arrivés à ma suite, **Ananya Ajith, Grégory de Bodt** et **Antoine Harvengt**, ont été une vraie bouffée d'air frais au sortir du COVID, et ont d'ailleurs eu à cœur de relancer la dynamique sociale du labo après de trop longs confinements. J'ai admiré Grégory pour son érudition et sa finesse d'esprit, Antoine pour son subtil mélange entre insouciance apparente et productivité ahurissante, Ananya for her courage and her determination to thrive in an environment so far from her confort zone, with a smile on her face. Enfin, j'ai regretté qu'**Antoine Delfosse** ne reste pas parmi nous plus longtemps, tant j'ai apprécié l'année partagée à ses côtés.

I would then like to sincerely thank the unique people that got me into "phage 101" at LabMCT. First, obviously, comes **Doctor Maya Merabishvili**, an unforgettable encounter and a phage master like no other; I loved her directness, her enthusiasm, her will to teach me to do things by myself, her sense of humor (that laugh !), and the food and drinks she brought back from vacation of course. I also thank **Johann Griselain**, **Christel Cochez** and **Griet Steurs** for their precious technical support. Enfin, bien sûr, je remercie chaleureusement le **Docteur Sarah Djebara**, pour nos collaborations si fructueuses, et nos interminables coups de fils à mi-chemin entre le débat scientifique et le commérage façon Desperate Housewives : j'en sors à chaque fois avec plein d'idées, et le sourire aux lèvres !

J'ai pu également compter sur l'aide de nombreux microbiologistes qui m'ont rendu de précieux services et ont tous contribué à entretenir mon intérêt pour cette discipline : à Saint-Luc, le **Professeur Hector Rodriguez-Villalobos**, le **Professeur Alexia Verroken**, le **Docteur Ahalieyah**

Anantarajah. En MBLG, le **Professeur Benoit Kabamba**, le **Docteur Jean Ruelle**, le **Docteur Géraldine Dessilly**, et **Eléonore Ngyuvula Mantu.**

De nombreux partenaires cliniques m'ont accordé leur confiance dans le traitement par phagothérapie de quelques patients dont nous nous souviendrons longtemps. Je voudrais remercier le **Docteur Olga Chatzis** en infectiologie pédiatrique, qui a été un soutien précieux depuis l'époque du mémoire, le **Professeur Pierre-Louis Docquier** en orthopédie pédiatrique, mais également et particulièrement l'équipe des soins intensifs pédiatriques avec qui j'ai eu le sentiment de nouer une relation de confiance précieuse, avec notamment à l'esprit le **Professeur Stéphan Clément de Cléty**, le **Docteur Laurent Houtekie**, et, bien sûr, avec le **Docteur Mathilde Balcaen**, qui sait à quel point j'ai aimé travailler avec elle sur un cas particulièrement complexe ces deux dernières années.

I would then sincerely thank a number of colleagues at KULeuven who greatly helped me in the design and technical realization of several assays, notably in bacterial variants sequencing and other bacterial phage resistance and phage-induced virulence tradeoffs investigations. **Professor Rob Lavigne**, **Doctor Jeroen Wagemans**, **Doctor Cedric Lood**, to name a few: thanks again!

Je voudrais remercier également ces quelques soutiens techniques ponctuels sans lesquels la thèse n'avancerait pas de la même manière, notamment l'équipe de l'hallucinante plateforme Winfab chez nos amis louvanistes ingénieurs civils : merci **Christian Renaux** et **Sébastien Faniel** de m'avoir accueilli dans cette soucoupe volante ! Mais également **Grégoire Emonts** bien sûr pour avoir été un fournisseur de PDMS à prix … imbattable,

Marc Kanbar pour le prêt de la pompe multi-channel qui m'a sauvé, **Arthur Colson** pour avoir vaillamment sacrifié tout un dimanche pour tenter un RNAscope pour le moins douteux qui s'est soldé par un glorieux et retentissant échec, **Cécile Boulanger** pour son aide en génomique WES, le **Professeur Françoise Van Bambeke** pour quelques doses d'antibiotiques dépannées et des conseils toujours avisés, l'équipe 2IP notamment **Aurélie Daumerie** (qui devait frôler l'infarctus à chaque fois qu'elle me voyait arriver avec un nouveau cathéter à microtomiser) et **Caroline Bouzin**, et l'équipe du MakiLab de Louvain-la-Neuve pour l'impression 3D. Thanks to **Wai Hoe Chin** for his incredibly detailed advise and his worthy experience in Gut-On-A-Chip design and handling.

Ces remerciements ne seraient bien sûr pas complets sans les adresser également au **Fonds de la recherche scientifique** (FNRS) et plus spécifiquement au **Fonds pour la formation à la recherche dans l'industrie et dans l'agriculture** (FRIA), qui ont rendu cette aventure possible de par leur soutien financier.

Mes derniers remerciements s'adressent, enfin, à toutes celles et ceux qui m'ont aidé sans doute sans le savoir, à mes proches, mes amis, mes colocs qui m'ont permis de me détendre quand il le fallait mais ont également fait preuve de compréhension et de patience quand je n'arrivais même plus à prendre le temps de répondre à leurs messages. À ma famille, enfin, et spécifiquement à mes parents qui, au fil des années, ont toujours eu à cœur de stimuler ma curiosité. Ils m'ont donné un cadre solide, tout en me laissant choisir les fils d'Ariane que je souhaitais suivre. C'est ici qu'ils m'ont mené.

Table of contents

Jury

Chair

Professor Isabelle Leclercq

Institut de Recherche Expérimentale et Clinique (IREC) – President

UCLouvain, Brussels, Belgium

Hepato-gastro-enterology Research Unit (IREC/GAEN)

Promotors

Professor Dimitri Van der Linden – promotor

Institut de Recherche Expérimentale et Clinique, UCLouvain

Pediatric Research Unit (IREC/PEDI)

Professor Leïla Belkhir – co-promotor

Institut de Recherche Expérimentale et Clinique, UCLouvain

Louvain center for Toxicology and Applied Pharmacology (IREC/LTAP)

Committee

Professor Pierre Smeesters

Department of Pediatrics, University Hospital Brussels

Université libre de Bruxelles, Belgium

Professor Piet Cools

Department of Diagnostic Sciences

Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium

Doctor Jean-Paul Pirnay

Laboratory for Molecular and Cellular Technology

Queen Astrid Military Hospital, Brussels, Belgium

Professor Etienne Sokal

Institut de Recherche Expérimentale et Clinique, UCLouvain

Pediatric Research Unit (IREC/PEDI)

Professor Xavier Wittebole

Department of Critical Care Medecine, Cliniques Universitaires Saint-Luc

UCLouvain, Brussels

Professor Thomas Michiels

Molecular Virology Unit, de Duve Institute

UCLouvain, Brussels

Summary

Antimicrobial resistance and more specifically bacterial resistance to antibiotics is a global threat for human health, and its burden is only expected to steadily increase in the coming decades. Complementary therapeutic tools are thus urgently needed in the fight against infections caused drug-resistant bacteria. Among them, phage therapy, the therapeutic use of lytic bacteriophage viruses, is likely the one to generate the most interest in recent clinical and fundamental research.

In this work, we related the use of phage therapy in seven patients who varied significantly in terms of demographical characteristics, clinical indication, phage therapy administration routes and outcomes. Through "reverse translational research", going from bedside to bench, we attempted to further characterize the factors likely to influence therapeutic success or failure. We suggested that complex mechanisms lying at different intersections between phages, antibiotics, bacteria and human patients might play crucial roles in that regard.

We also investigated the "carriage-infection conversion" paradigm of bacterial infections happening in the early post-operative period of pediatric liver transplantation recipients. This was allowed by a prospective study monitoring over 40 children awaiting liver transplantation, and by the use of a complex three-dimensional in-flow cell culture model, the Gut-On-A-Chip, to obtain preliminary insight in the potential of phage therapy to selectively decolonize the digestive carriage of specific bacterial species.

Résumé

La résistance aux antimicrobiens, et en particulier la résistance bactérienne aux antibiotiques, est une menace mondiale pour la santé humaine dont l'importance va certainement croitre dans les prochaines décennies. La recherche d'optiques thérapeutiques complémentaires aux antibiotiques est donc urgente, dans la lutte contre les infections causées par les bactéries antibio-résistantes. Parmi elles, la phagothérapie, qui consiste en l'utilisation thérapeutique de virus bactériophages, est sans doute celle qui génère le plus d'intérêt en recherche clinique et fondamentale récemment.

Nos travaux relatent l'utilisation de la phagothérapie chez sept patients variant significativement en termes de caractéristiques démographiques, d'indication clinique, de voie d'administration de la phagothérapie et de résultat thérapeutique. Plusieurs questions soulevées par ces cas ont fait l'objet de recherche translationnelle visant à mieux caractériser les déterminants de succès et d'échec thérapeutique. Nous suggérons que plusieurs mécanismes complexes à l'interface entre phages, antibiotiques, bactéries et patients y jouent probablement des rôles importants.

Nous avons également étudié le paradigme de "conversion portage-infection" dans les infections bactériennes post-opératoires précoces des enfants greffés de foie, notamment en menant une étude prospective suivant plus de 40 d'entre eux. Enfin, nous avons utilisé un modèle de culture tri-dimensionnelle en flux, un "Intestin-Sur-Puce", pour obtenir des données préliminaires quant à l'utilisation de phagothérapie en tant qu'agent de décolonisation digestive de portages d'espèces bactériennes spécifiques.

494

List of Abbreviations

aa	Amino acid
AB	Antibiotic
Ab	*Acinetobacter baumanii*
Abi	Abortive infection
ABOc	ABO-compatible
ABOi	ABO-incompatible
ABSSI	Acute bacterial skin and soft tissue infection
AMR	Antimicrobial resistance
AMX	Amoxicillin
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
ARG	Antibiotic Resistance Gene
ATMP	Advanced therapy medicinal product
AUC	Area under the curve
BAM	Bacteriophage Adherence to Mucus
BFC1	Bacteriofaagcocktail 1
BMP	Biological medicinal product
bp	Base pair
BPR	Bacterial phage resistance
BREX	Bacteriophage exclusion
BSI	Bloodstream infection
Bv	*Bacteroides vulgatus*
CBASS	Cyclic oligonucleotide-based anti-phage signaling system
CCV	Circular chromosomic view
CDC	Center for Disease Control
CDI	*Clostridium difficile* infection
CDS	Coding sequence
CFU	Colony forming unit
Ch	*Clostridium hathewayi*
CI	Confidence Interval
CI95	95% confidence interval
CIC	Carriage-infection conversion
CIP	Ciprofloxacin
CLN	Clindamycin
CMV	Cytomegalovirus

COG	Cluster of Orthologous Groups
COPD	Chronic obstructive pulmonary disease
COS	Cohesive end site
CPE	Carbapenemase-producing Enterobacterales
CPS	Capsular polysaccharide
CPT	Ceftaroline
CRO	Ceftriaxone
CRP	C-reactive protein
CST	Colistin
Ct	Cycle threshold
CTX	Cefotaxime
CTZ	Ceftazidime
CUSL	Cliniques universitaires Saint-Luc
cUTI	Complicated urinary tract infection
CWPS	Cell wall polysaccharide
DAO	Double agar overlay
DDLT	Deceased-donor liver transplantation
DISARM	Defense islands system associated with restriction-modification
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ds	Double-stranded
EBV	Epstein-Barr virus
Ec	*Escherichia coli*
EC	Ethics committee
ECM	Extra-cellular matrix
Ef	*Enterococcus faecium*
Efs	*Enterococcus faecalis*
EMA	European Medicine Agency
EOP	Efficiency of plating
EPS	Exopolysaccharide
Er	*Enterococcus rafinosus*
ESBL	*Extended-spectrum* beta-lactamase
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FBS	Fetal bovine serum
FLX	Flucloxacillin
Fm	*Finegoldia magna*
FMT	Fecal microbiota transplantation

gDNA	Genomic deoxyribonucleic acid
Gm	*Galleria mellonella*
GMP	Good manufacturing practices
GNB	Gram-negative bacilli
GOAC	Gut-On-A-Chip
GRADE	Grading of Recommendations Assessment, Development and Evaluation
HGT	Horizontal Gene Transfer
HSV	Herpes simplex virus
IA	Intra-abdominal
IAI	Intra-abdominal infection
ICU	Intensive Care Unit
IDSA	Infectious Diseases Society of America
IL	Intralesional
iMLSB	Inducible Macrolides-Lincosamides-Streptogramin B resistance phenotype
IP	Intra-pleural
IPA	Isopropyl alcohol
IPC	Infection Prvention and Control
IS	Insertion Sequence
IV	Intravenous
Kp	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* carbapenemase
LabMCT	Laboratory for Molecular and Cellular Technology
LB	Lysogeny broth
LDLT	Living-donor liver transplantation
LPS	Lipopolysaccharide
LT	Liver transplantation
LTA	Lipoteichoic acid
MALDI-TOF	Matrix Assisted Laser Desorption Ionization - Time of Flight
Mb	Megabase
MBP	Mucus-binding property
MDR	Multi-drug-resistant
MDRB	Multi-drug-resistant bacteria
MELD	Model for End-stage Liver Disease
MEM	Meropenem
MIC	Minimum Inhibitory Concentration
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-Resistant *Staphylococcus aureus*

MSSA	Methicillin-Susceptible *Staphylococcus aureus*
MSUD	Mapple syrup urine disease
MTZ	Metronidazole
nt	Nucleotide
OD	Optical density
OM	Outer membrane
OMV	Outer membrane vesicle
OOC	Organ-On-Chip
OPTICS	Oral Phage Therapy against Intestinal Carriage of Superbugs
Pa	*Pseudomonas aeruginosa*
PAGI	*Pseudomonas aeruginos*a genomic island
PAI	Phage-antibiotic interaction
PAS	Phage-antibiotic synergy
PBS	Phosphate-buffered saline
PDMS	Polydimethylsiloxane
PDR	Pan-drug-resistant
PFIC	Progressive familial intrahepatic cholestasis
PFU	Plaque forming unit
Ph	*Peptoniphilus harei*
PICU	Pediatric intensive care unit
PIN	Phage immune neutralization
PIP	Phage infection protein
PIVT	Phage-induced virulence tradeoff
Pm	*Proteus mirabilis*
PN	Pulmonary nebulization
PT	Phage therapy
PTM	Post-translational modification
PTMP	Phage therapy medicinal product
PTZ	Piperacillin-Tazobactam
PVL	Panton-Valentine Leukocidin
QAMH	Queen Astrid military hospital
RBP	Receptor binding protein
RCT	Randomized controlled trial
RIF	Rifampin
R-M	Restriction-modification
RNA	Ribonucleic acid
rRNA	Ribsomal ribonucleic acid
Sa	*Staphylococcus aureus*

SAP	Surgical antibiotic prophylaxis
SBD	Selective bowel decontamination
Sm	*Stenotrophomonas maltophilia*
SNP	Single Nucleotide Polymorphism
SOC	Standard-of-care
ss	Single-stranded
SSI	Surgical site infection
T4P	Type IV pili
T6SS	Type VI secretion system
TURP	Transurethral resection of the prostate
VAC	Vacuum-assisted closure
VAN	Vancomycin
VIM	Verona Integron-encoded Metallo-beta-lactamase
VRE	Vancomycin-resistant *Enterococcus*
VZV	Varicella zoster virus
WGS	Whole genome sequencing
WHO	World Health Organization
WT	Wild type
WTA	Wall teichoic acid
XDR	Extensively drug-resistant

Chapter I: General Introduction – Antimicrobial Resistance

I.1 General considerations and terminology

Antimicrobial resistance (AMR) is a global threat to human health. AMR theoretically designates the ability of (micro-)organisms (i.e. bacteria but also mycobacteria, viruses, fungi and parasites) to partly or fully resist to the action of one or several antimicrobial drug(s). Although some non-bacterial micro-organisms are commonly considered major threats in the scope of AMR, like *Mycobacterium tuberculosis* or *Aspergillus spp.* for example, bacteria are often considered to carry the majority of the burden caused by AMR, and the term AMR itself is often used as a synonym for its bacterial subset: antibiotic resistance[1]. In this work, we will focus on AMR in the sense of antibiotic resistance in bacteria.

AMR is generally considered to refer to the individual and specific acquisition of a resistant phenotype by a biological specimen belonging to a species that should normally be more susceptible to the considered antimicrobial agent. This can be referred to as "acquired" antimicrobial resistance. Conversely, AMR does not usually refer to the expected resistance or partial susceptibility to some antimicrobial agents contained in the "wild type" phenotype of a given bacterial species. This phenomenon is designated by a number of equivalent terms in literature, such as "intrinsic", "inherent", "innate" or "natural" resistance, or, more broadly, known as the wild type antimicrobial susceptibility profile of a given species. Examples of such

expected, innate antimicrobial resistance include cases where resistance is the consequence of a mechanistic incompatibility between antimicrobial mode of action and core bacterial molecular pathways or structural features. For example, polymyxins comprise a class of antibiotics that are known to exert most of their bactericidal effect by directly interacting with the lipopolysaccharides (LPS) included in Gram-negative bacteria's outer membrane (OM). Therefore, it seems logical that all Gram-positive bacteria are expected to be naturally resistant to colistin, as Gram-positive bacteria are structurally devoid of OM and, by extension, of LPS[2]. Such a scenario is thus not considered within the scope of AMR. There are, however, notable limitations and exceptions to such general rules, the discovery of these exceptions often leading to the investigation of secondary modes of action in a given antibiotic, and/or of structural or metabolic particularities in a bacterial species: for example, *Streptococcus pyogenes* is mostly susceptible to polymyxins although it is a Gram-positive cocci[2].

With the progressive emergence of combined acquired AMR, additional terminology was also needed to quantify the degree of antimicrobial resistance of a given bacterial specimen: to help this classification, international terms of multi- (MDR), extensively- (XDR) and pan- (PDR) drug resistant organisms have been defined and broadly used since 2011[3].

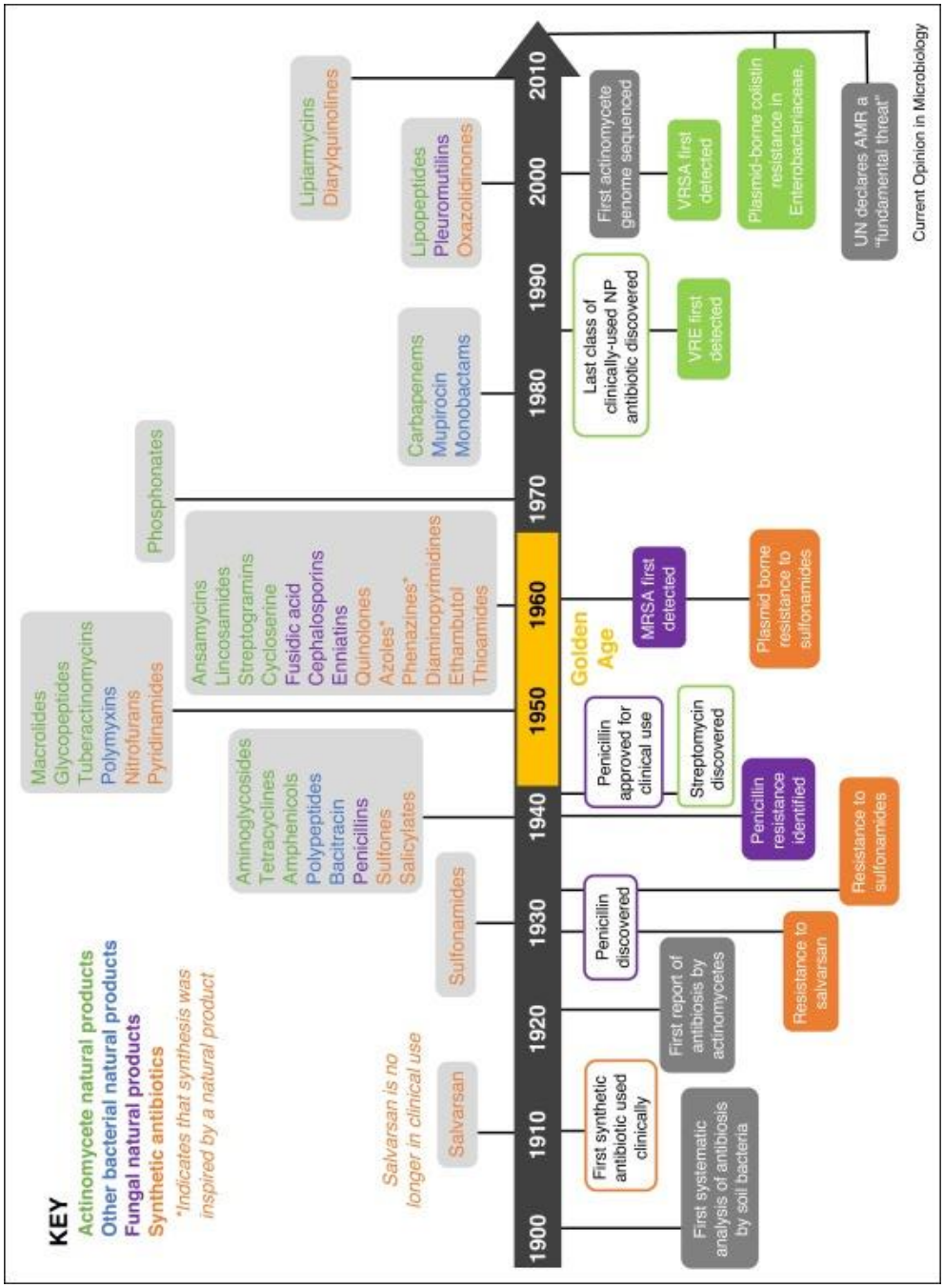
I.2 History

The history of AMR is inseparable to the history of antibiotics. The generic term "antibiotic" can be used to designate any organic molecule that either kills a given bacterial species or significantly impairs its growth, effects sometimes referred to as "bactericidal" or "bacteriostatic" respectively[4]. This definition is therefore a definition of function and not a definition of compound class or chemical structure, as antibiotics count many different classes of both naturally-occurring and synthetic molecular families[4].

The discovery of antibiotics and the consequences it had on the curative treatment of bacterial infections can be counted as one of the significant turning points for humanity that happened during the 20th century. It is commonly admitted that it participated, alongside increased hygiene and other key discoveries in the field of infectious diseases management such as vaccines, in the major increase in global life expectancy that happened during that century[5, 6, 7]. Early 20th century milestones in the development of antibiotics include the discovery and clinical use of salvarsan and Prontosil, mostly attributed to Paul Ehrlich and Gerhard Domagk respectively[8]. However, the founding event of this pharmacological success story is generally attributed to the discovery of penicillin by Alexander Fleming in 1928, though it would not reach commercial introduction and large-scale use before World War II. Following this starting point, antibiotic research and discovery knew a golden age during most of the mid-20th century, generating numerous new antibiotic compounds and classes in a comparatively short time interval (**Figure I.1**). This was however followed by "leaner years" in the 1970's and 1980's, as new antibiotic classes grew more and more difficult

to discover at the cost of increasingly complex and expensive technological constraints[4]. This downward trend would then only worsen around the turn of the century, showing a dramatic decrease in newly discovered antibiotics between the 1980's and early 2010's, at the expense of a significant increase in research efforts and budgets attributed to other fields of importance in the pharmacological industry, such as cancer treatments[6, 9].

587


KEY
Actinomycete natural products
Other bacterial natural products
Fungal natural products
Synthetic antibiotics
*Indicates that synthesis was inspired by a natural product
Salvarsan is no longer in clinical use
Salvarsan
Sulfonamides
Aminoglycosides
Tetracyclines
Amphenicols
Polypeptides
Bacitracin
Penicillins
Sulfones
Salicylates
Macrolides
Glycopeptides
Tuberactinomycins
Polymyxins
Nitrofurans
Pyridinamides
Phosphonates
Ansamycins
Lincosamides
Streptogramins
Cycloserine
Fusidic acid
Cephalosporins
Enniatins
Quinolones
Azoles*
Phenazines*
Diaminopyrimidines
Ethambutol
Thioamides
Carbapenems
Mupirocin
Monobactams
Lipopeptides
Pleuromutilins
Oxazolidinones
Lipiarmycins
Diarylquinolines
1900
1910
1920
1930
1940
1950
1960
1970
1980
1990
2000
2010
Golden Age
First systematic analysis of antibiosis by soil bacteria
First synthetic antibiotic used clinically
First report of antibiosis by actinomycetes
Salvarsan
Penicillin discovered
Resistance to salvarsan
Penicillin approved for clinical use
Penicillin resistance identified
Streptomycin discovered
Resistance to sulfonamides
MRSA first detected
Plasmid borne resistance to sulfonamides
Last class of clinically-used NP antibiotic discovered
VRE first detected
First actinomycete genome sequenced
VRSA first detected
Plasmid-borne colistin resistance in Enterobacteriaceae.
UN declares AMR a "fundamental threat"
Current Opinion in Microbiology

588

Fig. I.1 | Timeline showing the time new classes of antibiotics reached clinical use, as well as other key antibiotics-related historical events. The antibiotics are colored according to their source. From Hutchings et al., 2019[8]

Regrettably, this general decrease in antibiotic research interest, funding and results over the course of the second half of the 20th and beginning of the 21st century was mirrored by an opposite increasing trend regarding AMR development. Contrary to popular belief, the AMR problematic was not born in the 21st century, though the magnitude it has reached in recent years is admittedly unprecedented. Indeed, through all of antibiotic discovery's history, the comparative parallel timeline of first antimicrobial resistance detection has progressed in a desperately similar way. Time intervals between first introduction of a given antibiotic to the general use and first evidence of AMR acquisition against its effects indeed rarely exceed a few years, and no antibiotic class discovered so far has been fully spared from the dire phenomenon (**Fig. I.2**). Identification of resistant bacterial strains even sometimes precedes introduction of the antibiotic to general use, such as in the notorious case of penicillin: a resistance-conferring enzyme named "penicillinase" had already been identified in 1940, in the interval between penicillin discovery in 1928 and the start of its broad use around 1942-1943[4, 10]. The progressive discovery and characterization of additional members from the same enzymatic family, called Beta-lactamases, then skyrocketed between the early 1970s and late 2000's[4].

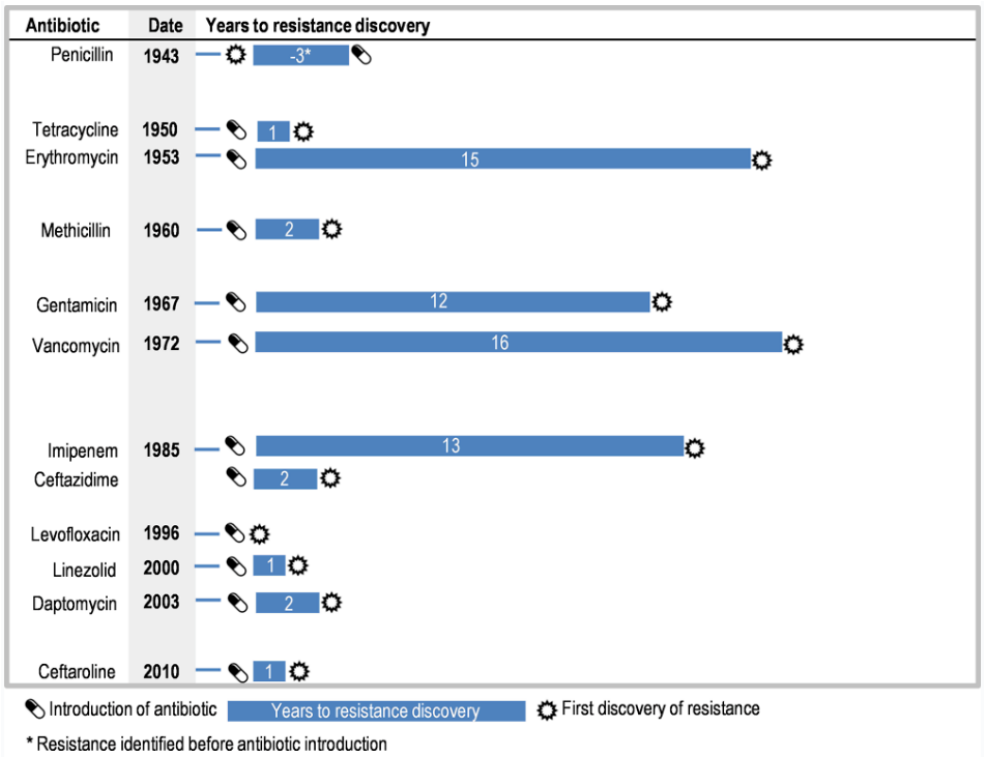


Fig. I.2 | Timeline of antibiotic discovery and detection of antibiotic resistance. From OCDE Health Policy Studies, "Stemming the Superbug Tide", 2018, adapted from Center for Disease Control (CDC) data, 2013, and Nelson & Levy, 2011[6].

The systematic identification of bacterial isolates exhibiting reduced susceptibility to an antimicrobial agent very early after the first introduction of said agent is still confirmed in recent notable examples, even when compounds exert exceedingly broad-spectrum bactericidal effect and rely on a first-in-kind mechanism such as in the case of cefiderocol, for example[11].

Besides the progression in qualitative understanding of these resistant phenotypes and their associated molecular structures and pathways, the late 20th and early 21st centuries most importantly faced a quantitative progression of the problematic as well. Indeed, the prevalence rate of resistant phenotypes among clinical isolates of numerous pathogens of high clinical relevance increased steadily during this period. This was typically documented by surveillance programs during the early 21st century in the hospital setting, focusing on notable resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), for example: this also illustrated that the increase in detection rates of such resistant isolates was not simply an epidemiological or micro-ecological phenomenon but translated in a tangible clinical problematic of increased rate of resistant infections (**Fig. I.3**)[12].

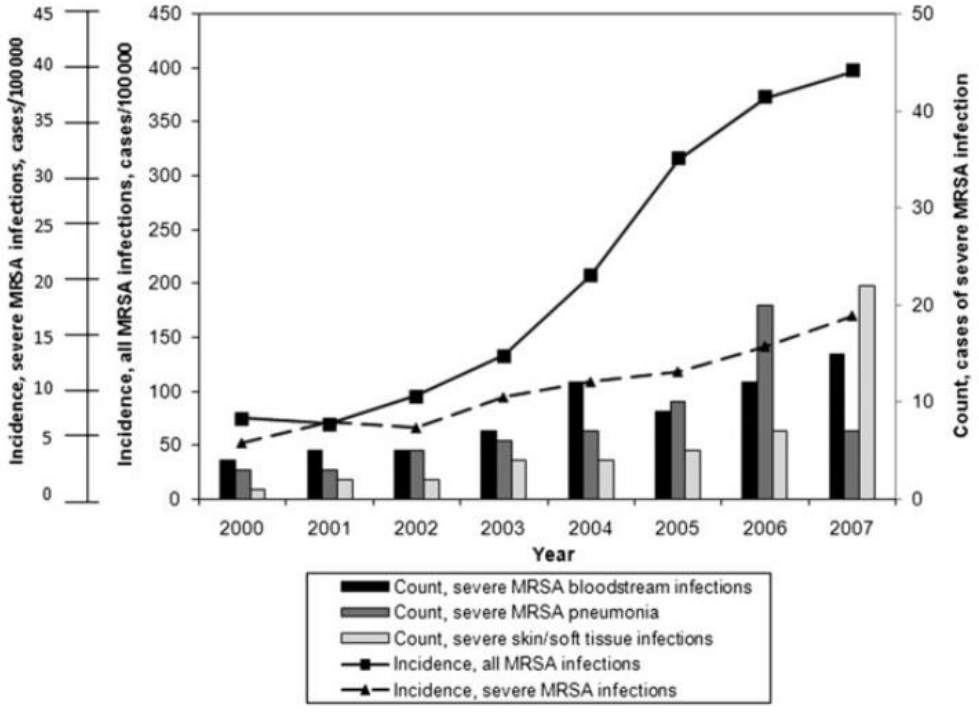
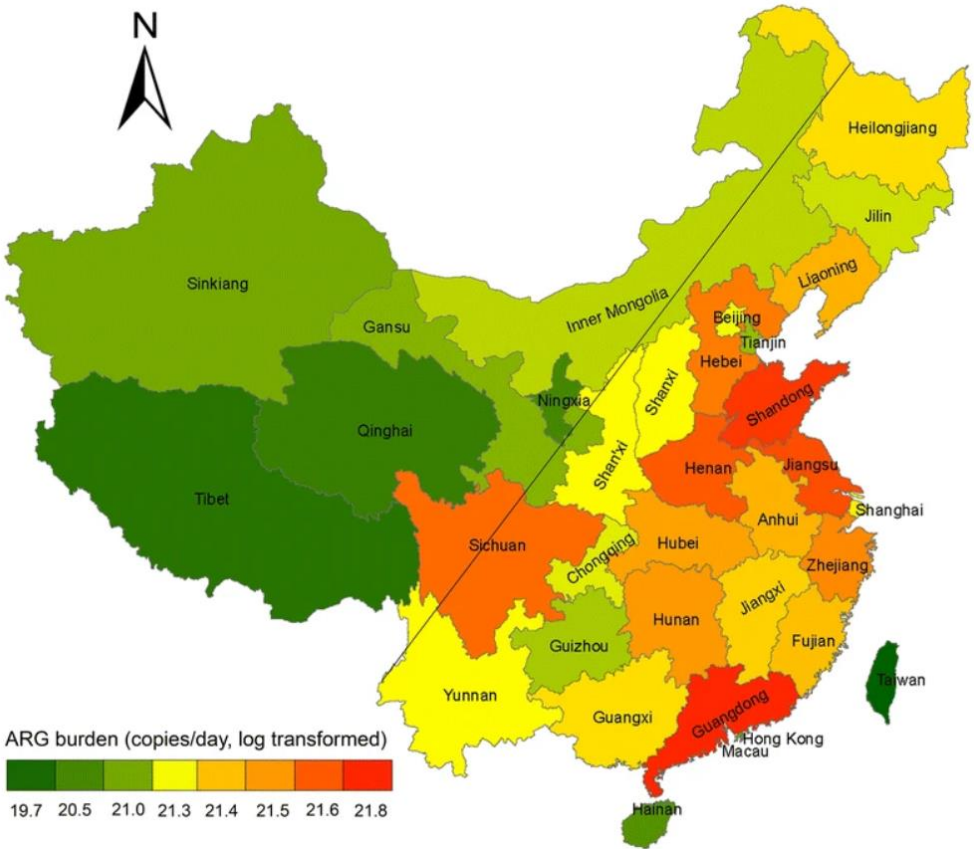


Fig I.3 | Trends in incidence of total and severe community-onset methicillin-resistant *Staphylococcus aureus* (MRSA) infections, John H. Stroger Jr (Cook County) Hospital, Chicago, Illinois. From Hota et al., 2011[12].

Over approximately the same timeframe, other works have shed light on very similar worsening dynamics regarding other resistant pathogens of major clinical importance such as vancomycin-resistant *Enterococcus spp.*, MDR/XDR *Pseudomonas aeruginosa*, or MDR/XDR Enterobacterales, for example[13, 14, 15].

I.3 Burden and contributing factors

While the AMR phenomenon has globally increased over the last decades as previously discussed, the situation today is however characterized by a striking geographical heterogeneity. Prevalence of resistant isolates among a given bacterial species can vary greatly from country to country, but also significantly from region to region inside a given country. This finding gave way to studies looking for various cofactors influencing the degree of locally-circulating AMR-related genes, such as demographics (**Fig. I.4**).



655

656 **Fig. I.4 | Antibiotic resistance genes (ARG) burden based on urban**
657 **populations of administrative districts in China.** The ARG burden in
658 Chinese administrative regions is calculated by multiplying the medium value
659 of ARG load (9.47×10^{13} copies/cap/day) by total urban population. The
660 contrasted repartition of ARG burden is strongly discriminated by the black
661 line representing the "Hu Huanyong line": the part east of this line containing
662 43% of China's surface area but 94% of its population[16]. From Su et al., 2017,
663 image by An Xin-Li[17].

664

Nevertheless, the most obvious and well documented aggravating factor identified in the rise of AMR has long been antibiotic consumption itself, both in human medicine and in agriculture, humans and animals being able to indirectly transmit resistant strains among or between them by the means of shared environmental reservoirs[18, 19, 20, 21]. As such, AMR is considered a prime example of a health problematic that should be considered under the "One Health" paradigm, defined as “the collaborative effort of multiple health science professions, together with their related disciplines and institutions—working locally, nationally, and globally—to attain optimal health for people, domestic animals, wildlife, plants, and our environment”: for example this implies an inter-dependency between human and animal health threats (e.g. zoonotic infections) and the need for a common, coordinated management of both of them to reach significant progress[22]. Extensive worldwide surveillance programs have documented that mean human antibiotic consumption per capita had increased globally in the last two decades, an likely had beforehand, though large data gaps exists in that regard[23]. In this context, antibiotic consumption, including over the counter use, acts as a powerful and short-term pressure lever for Darwinian selection in a literal "survival of the fittest" scenario. Previous works have, however, notably pointed out that increasing rates of resistance to a given antibiotic was not always primarily correlated to an increased use of the same antibiotic, but sometimes more strongly to the use of other antibiotics used to treat similar infections[24]. Other recent works have also underlined that the correlation-to-causality relationship of some of these alleged risk factors, notably agricultural antibiotic consumption, remained controversial[25].

In addition, researchers have progressively suspected this cause-consequence scenario of antibiotic consumption as the main (or even only) driver to the AMR crisis to be too simplistic. Accordingly, recent research has also revealed other major contributing factors to the spread of AMR such as demographical, socio-cultural, economical or even geophysical variables[25, 26, 27, 28]. Some recent works have focused on geographically mapping the discrepancies in this consumption-resistance paradigm, especially highlighting the double penalty of "low consumption, high resistance" in some low or middle income countries, advocating that antibiotic *misuse* might be a heavier AMR driver than antibiotic *overuse*[29]. The point was even made that gross antibiotic consumption was possibly not to be considered among the main AMR drivers, or at least that only working on lowering antibiotic consumption would probably fail at reverting the trend of global AMR spread[26]. This societal topic is thus very much subject to recent debate.

Regardless of its underlying factors of progression, AMR takes a massive toll on human health in current years, and this is only expected to worsen during the coming decades. Indeed, numerous retrospective worldwide research efforts have been aggregated to estimate that in 2019, approximately 1,27 million global deaths (Confidence Interval [CI] 95%: 0.911-1.710) have been directly related to infections caused by drug-resistant organisms (not only drug-resistant bacteria)[25]. As a comparison, this brings AMR-related deaths to a similar level as global road mortality, estimated around 1.35 million yearly deaths[30]. Among these AMR-related deaths, a single "drug-pathogen combination", MRSA, accounts for over 100 000 of these deaths alone. Authors have warned that despite being important in all world regions, this phenomenon was again characterized by an important

geographical heterogeneity, with most of the incurred mortality being found in Sub-Saharan Africa and South Asia. Dire predictive scenarios have been proposed by expert groups, the most famous one predicting an estimate of up to 10 million AMR-related yearly deaths by the year 2050, which could surpass cancer-related deaths, should no major action be taken to curb this trend by then[31]. These predictions have since then been debated, though the general consensus of a worsening trend seems to remain undisputed[32]. AMR also seemingly carries a significant - albeit also geographically heterogenous - economical cost, possibly due to its associated increases in length of hospital stay, morbidity, or rate of recurrent infection for example, though homogeneous prospective data is still needed to fully assess this aspect of the problematic[1, 31, 33, 34].

I.4 Mechanisms and pathogens of interest

The acquisition of an antimicrobial resistance phenotype at the bacterial cell level can be linked to the genetically- and/or epigenetically-mediated cellular expression of one or several mechanisms responsible for resistance[35]. These mechanisms can be schematically divided in four classes[36] **(Fig. I.5)**:

i) **Drug alteration**: the bacterial cell is able to alter the structure of the antibiotic compound, often by exerting an enzymatic activity, degrading the antibiotic and reducing its effect. Example: aforementioned enzymes from the Beta-lactamase family cleave the Beta-lactam ring, the active principle

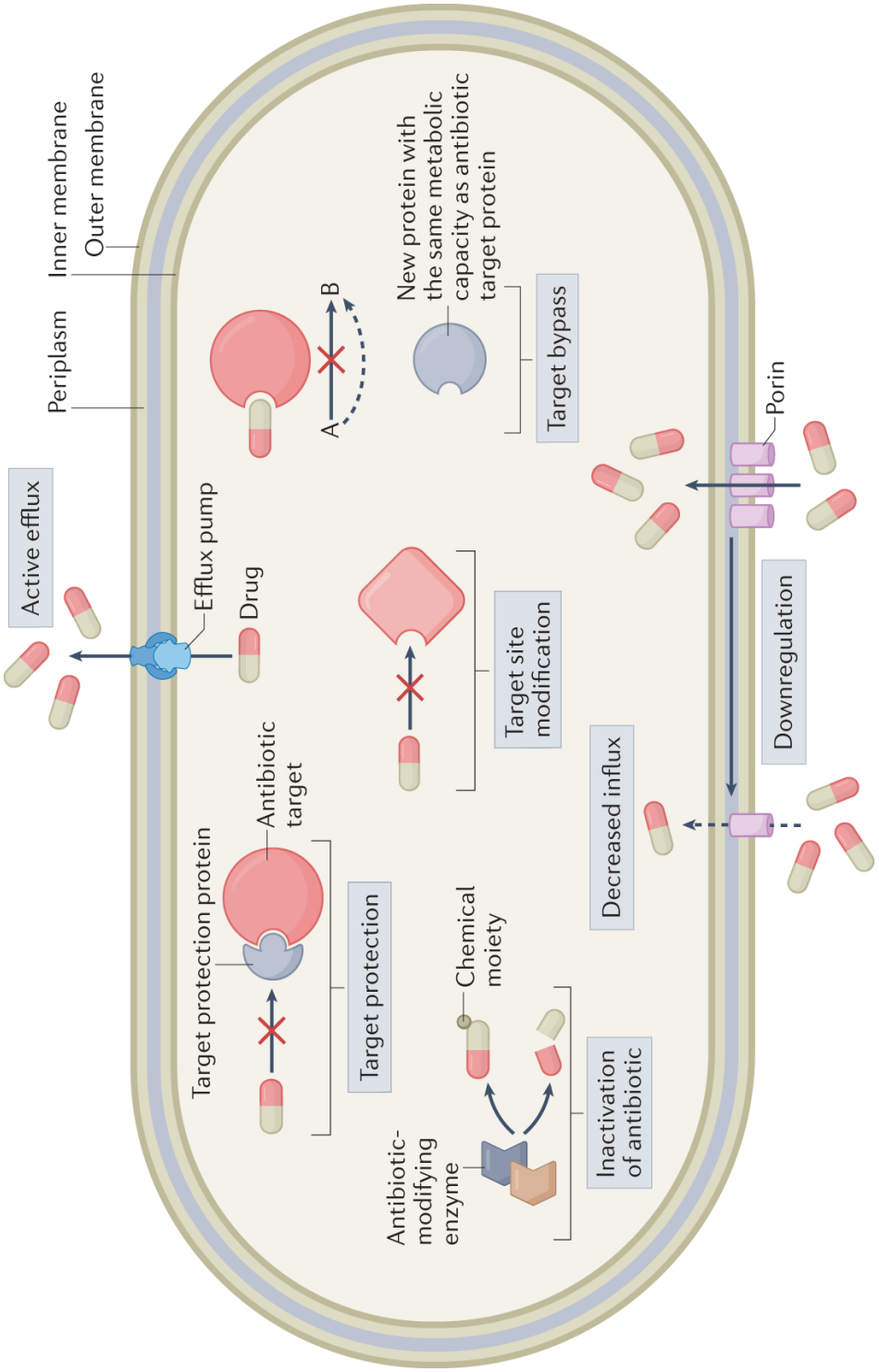
responsible for the bactericidal effect of numerous antibiotics (constitutive of the Beta-lactam antibiotic family).

ii) **Target modification / protection / bypass**: (one of) the molecular target(s) of the antibiotic compound is modified, or its access mechanically limited, so that the drug-target interaction required to obtain the antimicrobial effect is compromised. Example: single point mutation in DNA gyrase coding gene *gyrA* impedes its interaction with fluoroquinolones, reducing their effect[37].

iii) **Active efflux**: active energy-costing (multi-)efflux pumps located through the bacterial membrane(s) eject antibiotics from the intra-cellular to the extra-cellular space, preventing them from reaching their target. Example: *Pseudomonas aeruginosa* is able to express a variety of such efflux pumps to eject multiple families of antibiotics from its intra-cellular space such as aminoglycosides or fluoroquinolones, for example[38].

iv) **Uptake limitation**: initial penetration of the antibiotic compound inside the bacterial cell is limited. Example: inactivating mutation in *oprD* gene in *Pseudomonas aeruginosa* leads to a loss of porins in the bacterial outer membrane, which normally serve as entry points for carbapenem antibiotics, thus hampering their effect[39].

Some of these resistance mechanisms being more typically associated with certain classes of antibiotics[40].


Inner membrane
Outer membrane
Periplasm
Active efflux
Efflux pump
Drug
Target protection protein
Antibiotic target
Target protection
Antibiotic-modifying enzyme
Chemical moiety
Inactivation of antibiotic
Target site modification
A
B
New protein with the same metabolic capacity as antibiotic target protein
Target bypass
Porin
Decreased influx
Downregulation

762

Fig. I.5 | Schematic representation of the main classes of acquired antimicrobial resistance mechanisms. From Darby et al., 2023[41].

De novo acquisition of the genetic basis needed to express these mechanisms can also be schematically divided, this time into two fundamentally different processes of acquisition.

i) **Sporadic acquisition & vertical transmission**: spontaneous mutations occur randomly in the bacterial genome, and are then transmitted vertically during bacterial cell division from mother to daughter cells ; if the mutation confers a survival advantage in the considered context (e.g. an antibiotic-rich environment), it gets progressively selected. Examples of antimicrobial resistance acquisition by this pathway have been characterized for decades, and include for example the aforementioned case of single point mutation in *gyrA* conferring fluoroquinolone-resistance[42].

ii) **Horizontal Gene Transfer (HGT)**: a number of mechanisms allow mobile genetic elements of various sizes and structural nature to be transferred between two distinct bacterial cells, not linked by a mother-daughter relationship[43]. There is a wide consensus to consider HGT as being the most important acquisition pathway of the two and to be responsible for the massive, conjoined epidemic spread of multiple AMR genetic determinants in a given bacterial populations, but the exact characterization of HGT's contribution to the phenomenon might prove challenging[44, 45, 46]. HGT usually includes three canonical mechanisms[43] (**Fig I.6**):

- **Transformation**: the direct uptake of "free-floating" DNA fragments from the immediate micro-environment.

- **Transduction**: the mobilization of bacterial genetic elements by the successive integration and excision of a neighboring lysogenic bacteriophage virus genome.

- **Conjugation**: itself often considered to be the most important mechanism among HGT[43]. It consists in the donor-to-recipient transmission of a *conjugative* plasmid through a complex conjugation apparatus requiring pilus formation and direct cell-to-cell contact. In addition, conjugation can occur between phylogenetically-distant organisms.

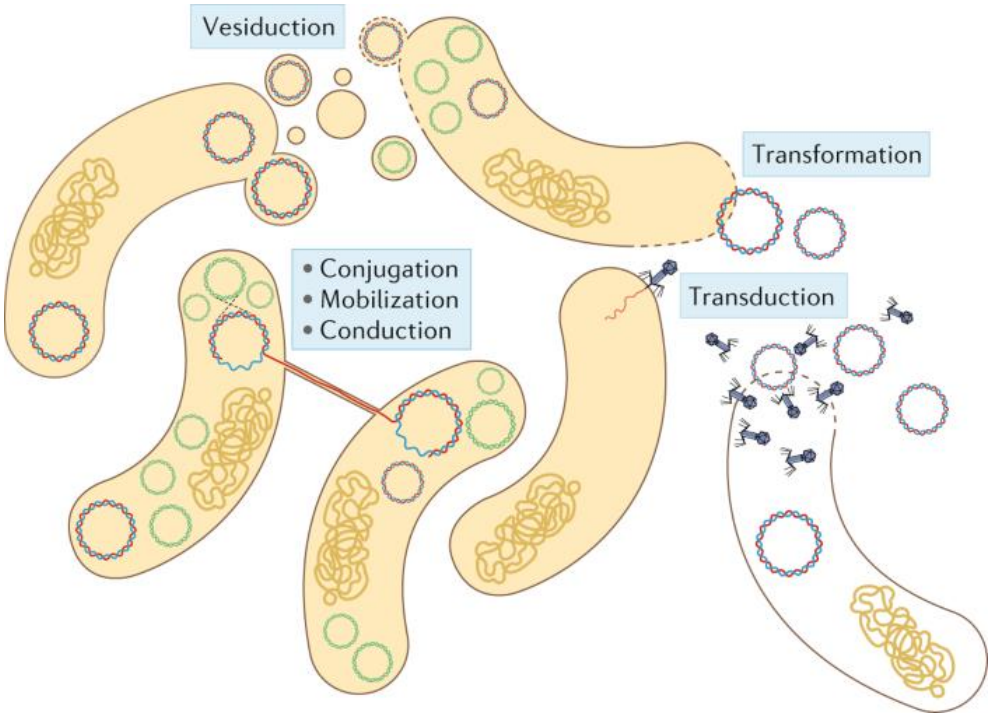


Fig. I.6 | Mechanisms for horizontal gene transfer of plasmids. From Rodriguez-Beltran et al., 2021[43].

Of note, mobilization of non-conjugative plasmids and even conduction of technically non-mobilizable plasmids by recombination and co-integration with a mobilizable plasmid are considered sub-mechanisms of conjugation. A new sub-mechanism of transduction has also been recently characterized and termed lateral transduction, in addition to the previously described generalized and specialized transduction[47]. It brings a unique contribution to the mobility of bacterial chromosomic genetic elements, previously thought to be intrinsically much less mobile than their non-chromosomal counterparts[48]. Lastly, vesiduction, the transmission of plasmids by secreted vesicles, has recently been identified as a fourth non-canonical mechanism of HGT, believed to occur at high rate in biofilms[49, 50].

As a general remark, we should mention that the spread of AMR genetic determinants should however not be simplified to the concept of a succession of distinct direct cell-to-cell transfers. Rather than appreciating it at this individual level, sustained HGT should be considered at the macroscopic community level, where richly diverse bacterial communities are together constitutive of a global shared pool of such genes, termed the resistome, that they can use according to community-driven needs [51, 52]. Such a global collection of genes and the mobility dynamics it fosters predates antibiotic use by millennia and is merely shaped by it, not caused by it[53]. Our own healthy microbiota is thus also home to part of this resistome, and pathogenic bacteria in contact with it are thought to be able to take advantage of this vast gene library, making these seemingly beneficial microbial communities silent contributors to the spread of AMR[44].

The threat level caused by the rising AMR problematic is not homogeneous across bacterial species. Though various recent works have come to similar conclusions, the exact content of the list of pathogens deemed of critical priority regarding AMR threat has been subject to debate. Fifteen years ago, aggregated data from various hospital-based surveillance programs as well as from the Infectious Diseases Society of America (IDSA) led to the proposition of the ESKAPE group, a now famous acronym encompassing the following bacterial species deemed to be top-tier pathogens in antimicrobial-resistant nosocomial infections[54, 55] :

- *Enterococcus faecium*
- *Staphylococcus aureus*
- *Klebsiella pneumoniae*
- *Acinetobacter baumanii*
- *Pseudomonas aeruginosa*
- *Enterobacter* species

Since then, other research groups have proposed other lists of priority, at times pointing out discrepancies between them. An example of such differences can be found in the discussion of the benchmark 2019 systematic analysis of global burden of bacterial antimicrobial resistance, where the authors, forming the "Antimicrobial Resistance Collaborators", regret that World Health Organization (WHO)'s 2017 list (**Fig. I.7**) did not include fluoroquinolone-resistant *Escherichia coli*, or that it separated MDR *Mycobacterium tuberculosis* from the rest of the priority list, or also that

MRSA would only be considered a pathogen of "high" but not "critical" importance despite its aforementioned leading role in global AMR-related mortality[25]. Nevertheless, as these extensive works aim at influencing policymakers and researchers in the quest for a better handling of the AMR crisis, it seems logical that these lists would be meant to evolve in the coming years, for example if significant short-term action was able to mitigate the threat caused by one of these pathogens, like it might be the case with *Streptococcus pneumoniae* vaccination, for example[25].

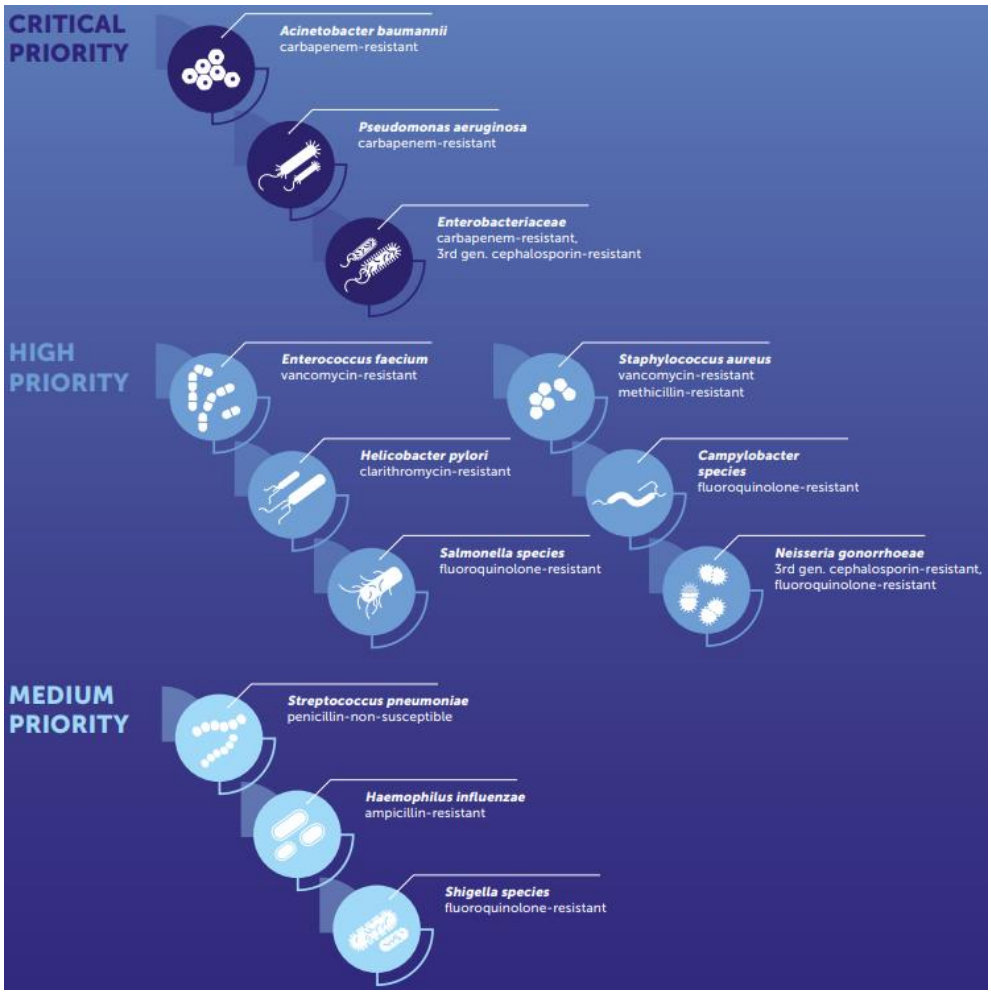


Fig. I.7 | "Priorization of pathogens to guide Research and Development of new antibiotics", WHO 2017[56].

I.5 Conclusions and current perspectives

The paradoxical combination, during the late 20th and early 21st centuries, of an increasing global antibiotic consumption and a decreasing trend in new antibiotic research and development has constituted an enormous

selective pressure lever on a largely pre-existing global "resistome", allowing highly pathogenic bacteria to develop multiple concomitant antimicrobial resistance mechanisms. Infections caused by these bacteria now frequently lead to therapeutic hurdles or even dead-ends, which translate into a significant and specific mortality of over 1.2 million yearly deaths. This general summarized phenomenon is worsened by added layers of complexity, with newly found risk factors of demographical, economical, socio-cultural or even geophysical nature. This specific part of the problematic creates a striking geographical heterogeneity often leading to "double penalty" scenario in low or middle income countries. The quality of surveillance programs themselves also proves very heterogenous, which in turn hampers the precise monitoring of the problematic in the coming times[34].

Now that the main lines of the problematic have been drawn, it seems logical that the next step would be the search for solutions.

A number of preventive recommendations have been issued by aforementioned international research consortia[25, 57]. These include increased preventive measures to contain the spread of infections such as increased infection prevention and control (IPC) programs, including sanitation- and hygiene-related measures and vaccination, but also re-insist on the necessity of limiting antibiotic use to situations that necessitate it, avoiding empirical antibiotic treatment for likely viral respiratory infections for example: nationally-coordinated programs such as Sweden's "Strama" program have suggested that such approaches could generate positive impact on local AMR[58]. Of important note, while these recommendations generally insist on the necessity of reinvesting in the development of new antibiotics, they also

clearly state it as one among a list of numerous measures, implying that such action would likely be necessary but not sufficient at this stage.

Research and development of new antibiotics has recently known a moderate regain of interest to face the urge, in part thanks to increased public investments[59]. A number of new antibiotics have reached market approval in the last few years **(Table I.1)**, and others are in various clinical or pre-clinical development stages[59, 60]. Validated indications for recently approved antibiotics remain however limited, and reasonably redefining these indications to a larger panel without putting patients in poor benefit-risk ratio situations is a newfound challenge in antimicrobial clinical research[61].

Gram-positive bacteria

class	Representative drug	New derivatives
Beta-lactams	oxacillin	ceftaroline
glycopeptides	vancomycin	telavancin, oritavancin, dalbavancin
fluoroquinolones	moxifloxacin	delafloxacin
oxazolidinones	linezolid	tedizolid

Gram-negative bacteria

class	Representative drug	New derivatives
Beta-lactams (+ Inhibitors)	ceftazidime, meropenem	ceftolozane-tazobactam; ceftazidime-avibactam; imipenem-cilastatin-ralebactam meropenem-varbobactam cefiderocol
aminoglycosides	gentamicin	plazomicin

Others

class	Representative drug	New derivatives
Pleuromutilins	tilmicosin (veterinary)	lefamulin
Tetracyclines	doxyxycline	tigecycline, omadacycline,eravacycline

Table I.1 | Examples of recently approved antibiotics by main Gram-defined target. Courtesy of F. Van Bambeke, 2021. Based on the exhaustive 2022 report published by the World Health Organization[60].

Many other research works have however called for alternatives or complementary therapeutics to antibiotics, underlining the daunting difficulties of new antibiotic development, and the poor return on investment that many of these new development programs have yielded, sometimes at the cost of important public money investments[62, 63, 64, 65].

These potential complementary therapeutics have (re-)gained some interest in recent research, and are at various stages of pre-clinical or clinical experimentation at the moment. Among them can be briefly cited antimicrobial peptides[66], antimicrobial antibodies[67, 68], anti-virulence therapies[69], quorum-sensing inhibitors[70], *Bdellovibrio bacteriovorus*-based therapy[71], for example.

In this work, however, we decided to focus on one of those complementary therapeutic options, the one that likely possesses the most extended fundamental and pre-clinical research experience as well as the most opportunities for clinical use: phage therapy.

928

929

930

931

932

933

934

935

936

937

938

939

940

941

942

943

944

945

Chapter II: General Introduction – Bacteriophages and Phage Therapy

II.1 Foreword

A practical definition of phage therapy (PT) could be: the exploitation of the **lytic cycle** of some **bacteriophage viruses** for antibacterial **therapeutic purposes**.

This chapter will be articulated around the development of the three bolded terms of this proposed definition:

- *Bacteriophage viruses:* section II.2

- *Lytic cycle*: section II.3

- *Therapeutic purposes*: section II.4

II.2 *Bacteriophage viruses*

II.2.1 General considerations and historical perspective

Bacteriophage viruses, or phages for short, are viruses specifically and exclusively infecting bacteria: their replication host cell must be a bacterial cell.

The first historically reported observation of phages, or rather of their effects on bacterial culture, is independently attributed to two scientists from the early 20th century: Frederic Twort (London, 1915) and Félix d'Hérelle

(Paris, 1917)[72, 73]. Previous observations, twenty years beforehand (1896), of the bacteriolytic properties of some Ganges and Junma rivers water samples by English bacteriologist Ernest Hankin are frequently cited as potential early observations of phage effect: though it has for some time been believed by d'Hérelle himself, this has nevertheless been challenged in recent research, and is generally not considered a dispute of Twort and d'Hérelle's paternity[74]. In the early days of his discovery, d'Hérelle found out that filtrate of stools coming from dysenteric patients showed "antagonistic properties" against the causative bacteria, creating lysis plaques on bacterial cultures[75]. He hypothesized that these antagonistic properties were mediated by putative filtrable viruses ("ultraviruses") from these stool samples. In the absence of electron microscopy at that time, the viral nature of these particles could only remain hypothetical: this definite microscopical observation would happen in 1940[76]. Nevertheless, he went on to illustrate that titers of these antagonistic agents increased during infection and peaked upon recovery, potentially suggestive of a replicating nature of the hypothetical agent to the detriment of bacterial prosperity.

Shortly after his first discoveries, d'Hérelle sensed that the potential of phages exceeded fundamental microbiology. He quickly developed an interest in phages as anti-infectious agents. Emile Roux, inspired by the clinical tradition of the Institut Pasteur he was directing at the time, immediately supported the experimental investigation of the idea of PT, initially on cases of fowl typhoid caused by *Salmonella enterica* Gallinarum in hens, then on dysentery caused by *Shigella dysenteriae* in rabbits[77].

These first experiments proved encouraging, and post-hoc analysis of d'Hérelle's research suggests that his methodology was consistent with the scientific quality standards of the time. However, these did not yet include double-blind analysis[77].

The first human attempts at PT were planned from as early as 1919. D'Hérelle wanted to illustrate its safety in humans: he thus decided to self-ingest and then even self-inject increasing quantities of phage solution, without noticing any adverse events on himself. When subjected to the same protocol, members of his family and a number of colleagues did not reveal any either. Following this, D'Hérelle obtained the authorization to carry out the first human phage administration for therapeutic purpose in a sick patient: an oral anti-*Shigella* PT in a series of culture-positive bacillary dysentery. Shortly afterwards, d'Hérelle was credited with curing four cases of bubonic plague in Egypt by *in loco* PT (intra-bubonic injections). The episode was described as "remarkable" in the scientific community: human PT was already gaining its first credentials[77, 78]. D'Hérelle travelled the world during the late 1910's to the mid 1930's, fighting epidemics of plague and cholera in China, Laos, Vietnam, India, and several African countries in what can be considered the golden age of PT[78].

However, these promising early developments were followed by a progressive decline. In 1928, as we mentioned earlier, the antibiotic era begun with the discovery of penicillin by Fleming. During the mid-1930's, d'Hérelle went into exile in Georgia, where he worked in what is now known as the Eliava Institute, a reference center for phage research. It had previously been founded in 1923 by George Eliava, another key phage researcher of the early

20th century who d'Hérelle had previously met and befriended in Paris' Pasteur Institute. These Georgian years put d'Hérelle further from the Western scene. During these same years, the Council on Pharmacy and Chemistry in the USA decided to issue a formal statement on PT. Firstly, it presented the results of the combined literature as mixed, mentioning not only well-known successes but also clear failures. Secondly, it insisted on the worryingly non-standardized nature of phage preparations, which had not yet reached their industrial age[77, 78]. Penicillin might appear as a solution to both of these complaints.

For these initial reasons, the use of penicillin during the Second World War supplanted that of PT among American troops and the majority of European troops. PT was nevertheless massively used on the battlefield among Soviet troops, notably during their so-called "Winter War" against the Finns. Once World War II was over, however, ideological tensions grew between the West and the Soviets: PT was from then on politically and ideologically tainted, which became the final factor of demise for PT in the West. In addition, the first cases of bacterial resistance to phages were beginning to be observed, raising doubts about the long-term reliability of PT[75, 77]. For all these reasons, PT would virtually disappear in the West after World War II, whereas it subsisted in some ex-Soviet countries, notably Georgia thanks to the persistence of the Eliava Institute.

It is only in the mid 2000's to early 2010's that phage research and PT more specifically would face a massive regain of interest in the light of the aforementioned antimicrobial resistance crisis, which can still be considered today a "second golden age" in the field[79, 80]. Belgium was able to get on board

in due time, notably thanks to the early interest for PT of the Queen Astrid Military Hospital's Laboratory for Molecular and Cellular Technology (LabMCT).

II.2.2 Production and regulation

Aside from exceptional cases, phages used in PT are not the product of synthetic biology or any form of genetic engineering[81]. Instead, PT relies on the use of naturally-occurring phages, which can be considered bacteria's natural predators, found in various microbiologically-rich environmental samples such as sewage water. From these primary samples, phages are cultured on surrogate host bacterial strains and endure steps of isolation-propagation, phenotypic characterization (morphological by the use of electron microscopy but also functional including host range and plaque morphology), filtration, endotoxin purification, and then typically when considered for therapeutic use, genomic characterization[82].

Phages can then be used alone in a targeted fashion (see next section) or, like it has mostly been the case during the 20th century, as multi-phage cocktails of either fixed or evolving composition: many intermediate situations exist, all with their advantages and shortcomings[83]. Most modern western groups however might favor the targeted, "tailored" way of personalized PT instead of the "one-size-fits-all" scenario of fixed multi-phage cocktails, though they might be complementary. This is in part due to the possibly increased flexibility of personalized PT in the current industrial and regulatory framework **(Fig. II.1)**[84].

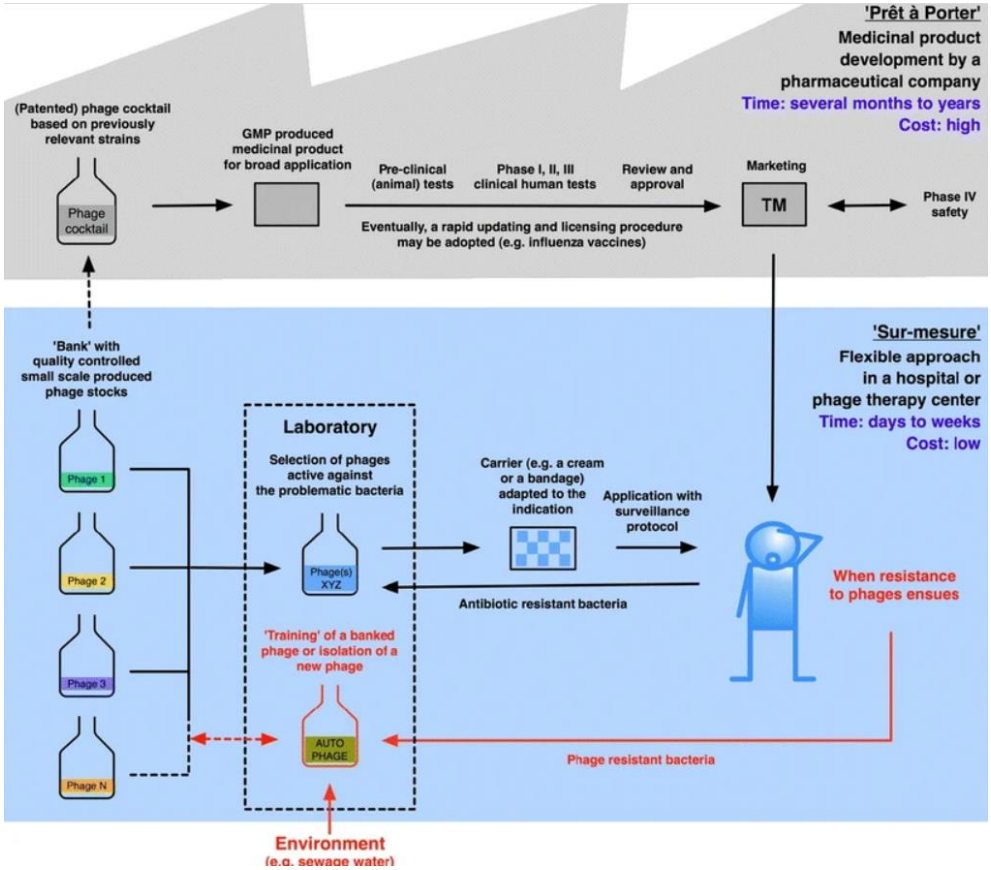


Fig. II.1 | "Phage therapy concepts: *prêt-à-porter* or *sur-mesure*?". Personalized phage therapy appears as a flexible application provided a close phage laboratory-hospital collaboration exists, whereas fixed composition phage cocktails, destined for empirical use and taking the route of canonical industrial production and experimental validation, might suffer from the intrinsic inertia of this process. From Pirnay et al., 2011[84].

This situation mostly reflects the elusive pharmaco-legal status that phages and PT have been granted in recent times. To only mention the European situation, phages have long been classified by the European

Medicine Agency (EMA) as *medicinal products*, though their specific affiliation to either biological medicinal products (BMP) or advanced therapy medicinal products (ATMP) has been subject to debate[84, 85, 86]. They are nowadays considered under the umbrella of BMP, sometimes more specifically being referred to as phage therapy medicinal products (PTMP)[86, 87, 88]. Recent advances have been made at the EMA level on the definition of dedicated regulatory guidelines for PT in the veterinary field (EMA/CVMP/NTWP/32862/2022), fostering hope for new similar endeavor in human therapy in a near future.

Regardless of this regulatory status though, phages considered as medicinal products theoretically must abide by the rules regulating their use, which include the need to obtain market authorization before routine use. This brings the problematic back to the shortcomings of the aforementioned "prêt-à-porter" phage paradigm. This would indeed require the time- and cost-intensive individual validation of each multi-phage combination considered for use, which is often deemed unrealistic besides being incompatible with an adaptable, personalized therapy perspective[84, 89, 90].

Despite these regulatory constraints, Belgium has proposed a dedicated regulatory framework for practical PT use at the national level, exploiting the single paradigm in which medicinal products can access a legitimate "routine" drug status without requiring market authorization, the magistral preparation[90, 91]. Indeed, after combined work between the national scientific community and regulatory authorities, Belgium has extended the list of medicinal products allowed into magistral preparations to phages, at the three following conditions:

- As they do not meet the requirements of European, Belgian or other national pharmacopeia, phages destined to the making of human PT magistral preparations must be produced by a Belgian national-approved laboratory on that regard (i.e. LabMCT) under the form of active pharmaceutical ingredients (API), following the instructions of an official monograph, and must obtain "green light" validation by the relevant national institute (i.e. Sciensano, the Belgian scientific institute of public health) regarding both phage genomic characterization and API batch safety (i.e. endotoxin quantification for example).

- Based on these API, the PT magistral preparation must then be prepared by a hospital pharmacist based on a nominal prescription destined to a single identified patient

- Finally, the PT magistral preparation must be administered by a hospital physician under its direct responsibility.

Other countries have since then taken interest in this pathway as well, though recent literature highlights that many different models co-exist to this day, even inside the European Union alone[90]. Countries devoid of such a dedicated framework still must rely on Article 37 of the Declaration of Helsinki regarding "unproven interventions in clinical practice" to administer PT in an often-called "compassionate" fashion[92].

Regardless of PT's ease of use in a given specified patient under the "Magistral Phage" paradigm, this *sui generis* framework does not, however, ease its use in approved clinical trials. Indeed, the use of PT products in this case automatically labels phages as "investigational medicinal products", a

status which necessarily implies the observance of a number of technical specifications. Among them is the paramount obligation to be produced in a 100% Good Manufacturing Practices (GMP)-compliant way, which phage APIs produced by LabMCT are officially not. This regulatory constraint specific to clinical trials creates a paradoxical situation in which patient-tailored PT can be legally applied in "routine" clinical practice (given the aforementioned conditions are respected), outside of any experimental or compassionate context and potentially more akin to a "standard-of-care" (SOC) treatment, whereas formal standardized investigations addressing the same clinical indications and problematics are incomparably more difficult to carry out. This likely disproportionate application of the GMP-centered paradigm to phage clinical research has been the subject of frequent criticism in literature[90]. European authorities might accordingly reconsider the situation, though initial projects proposed on that regard do not seem to have significantly evolved in the last ten years[93].

II.3 *Lytic cycle* – phage biology introduction

II.3.1 Modern taxonomy

At over 10^{30} worldwide viral particles and over 6000 distinct members observed by electron microscopy since the mid-20th century, phages are likely to be the largest virus group known[94]. Their taxonomy has however been subject to regular modifications. Up to the 2010's, classification based on morphological findings of aforementioned electron microscopy observations prevailed. Bacteriophages were all related to a single recognized viral order, Caudovirales, and numerous other phage families **(Fig. II.2)**[95].

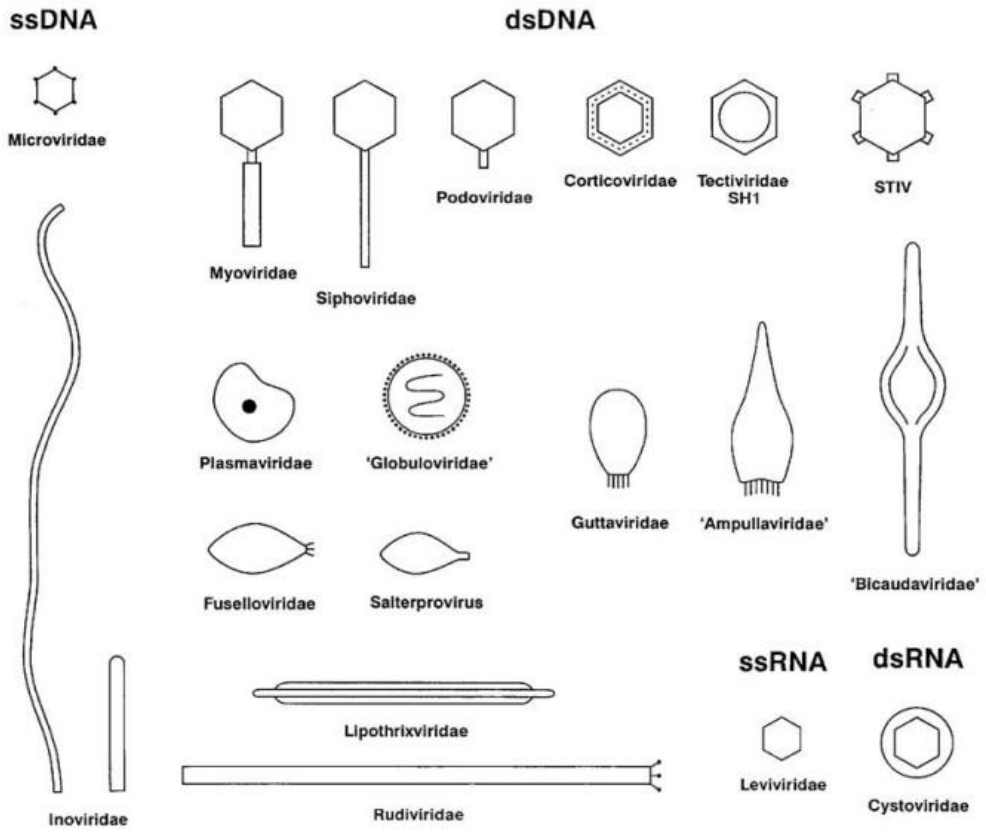


Fig. II.2 | Historical morphological phage taxonomy, notably developed and popularized by the late Hans-Wolfgang Ackermann (here from 2009)[95]. *ds, double-stranded; ss, single-stranded; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.*

The vast majority of these phages were comprised in the three subfamilies constitutive of the Caudovirales order or so-called "tailed phages", all harboring dsDNA genomes and no envelopes: *Myoviridae*, characterized by a contractile tail, *Siphoviridae*, characterized by a long, thin, non-contractile tail, and *Podoviridae*, characterized by a short, sometimes virtually absent tail. These three historical phage families have long been the exclusive phages used in conventional PT.

However, recent research groups have pointed out that such morphological taxonomy was outdated, as it proved polyphyletic when confronted to modern genome-based characterization of its families and members. Based on this fact and in collaboration with Ackermann himself, one of these groups has recently proposed to abolish the Caudovirales order and its iconic three "families", instead advocating for the creation of new genome-based classes, orders and families in accordance to modern taxonomical standards[96]. In our experience, this evolution has been relatively well received and is progressively implemented in the phage research community, in particular because the proposing authors recognize that the residual use of terms like "Myovirus" or "Podovirus" still retains its practical descriptive value[96].

II.3.2 Lytic cycle

Phages used in PT are "strictly lytic" phages, meaning their intra-bacterial replication cycle always and exclusively results in a "productive" cycle, synonymous of progeny synthesis (*de novo* creation of "daughter" phages) which are then released upon the phage-triggered lysis of the bacterial host cell. The steps of this cycle have long been described, and can be summarized as follows **(Fig. II.3)** [97, 98, 99]:

- **Adsorption**: reversible then irreversible attachment of the bacteriophage tail end to the bacterial cell surface, mediated by the specific recognition and binding of the phage's receptor binding protein (RBP) to its cognate bacterial surface receptor. This phase is optionally preceded by bacterial capsule degradation.

- **Viral genome intra-cellular injection** following surface peptidoglycan degradation.

- **Transcription, translation and replication** of the free-floating, non-integrated viral genome, by exploiting the enzyme-ribosomal bacterial machinery.

- **Assembly and packaging** of these replication and translation products into functional progeny virions

- **Virion-originated endolysin secretion** resulting in the host bacterial cell membrane lysis and the expulsion of the new virions in the extracellular environment.

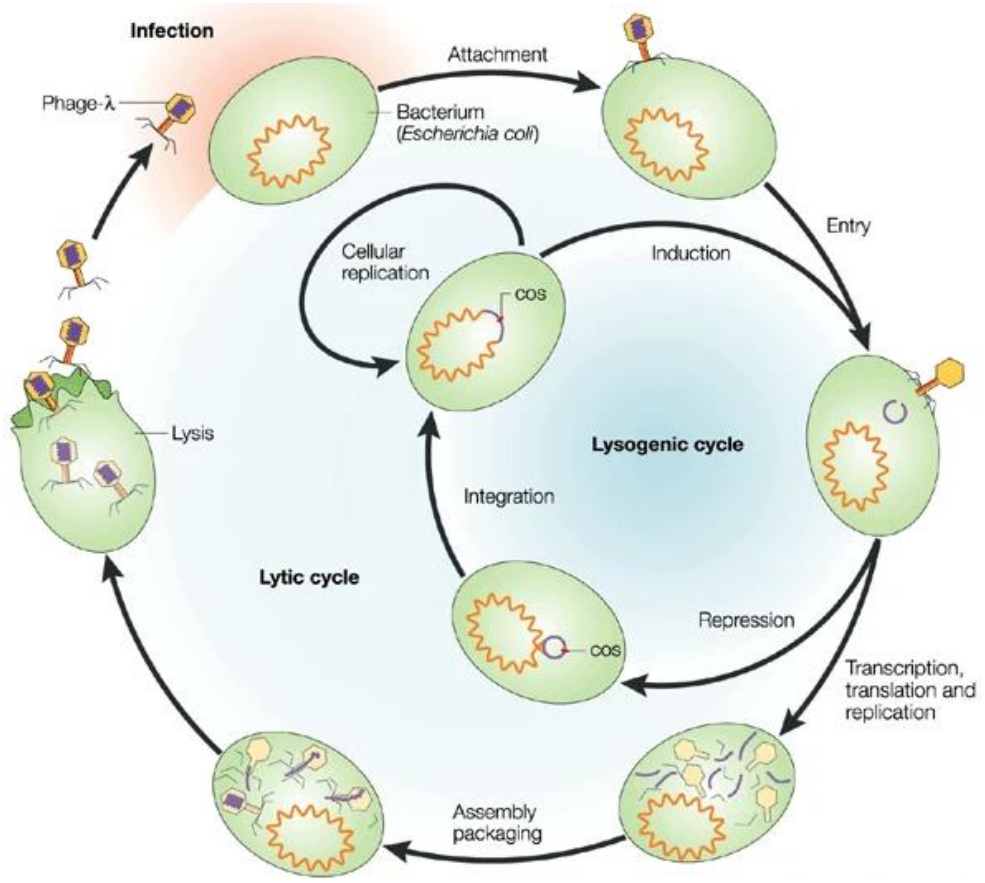


Fig. II.3 | Phage lytic and lysogenic cycles. Here exemplified by coliphage lambda and *Escherichia coli*. Lytic cycle consists in five major steps: attachment/adsorption, entry/injection, transcription-translation-replication, assembly, lysis. Conversely, lysogenic cycle reproduces the same first two steps but then switches to a defining phase of viral genome integration inside the bacterial host genome, a phenomenon that can be reversed under certain circumstances to excise and re-circularize phage genome. Strictly lytic phages used in phage therapy are carefully selected for their incapacity to perform such a lysogenic cycle. *COS, cohesive end site, point of closure after lysogenic viral genome integration.* From Allan Campbell, 2003[98].

The first step of this cycle, phage adsorption, requires recognition and binding of a given bacterial surface receptor to the phage's RBP located at its tail end. Such recognition is one among a series of key features defining phages' infective specificity, and narrow bactericidal spectrum in the context of PT[100, 101, 102]. Previous works have identified an array of typical bacterial surface receptors in both Gram-positive and Gram-negative bacteria, which include but are not limited to structures able to act as virulence factors for these bacteria, which will be re-addressed in Chapter III **(Fig. II.4)**[101, 103].

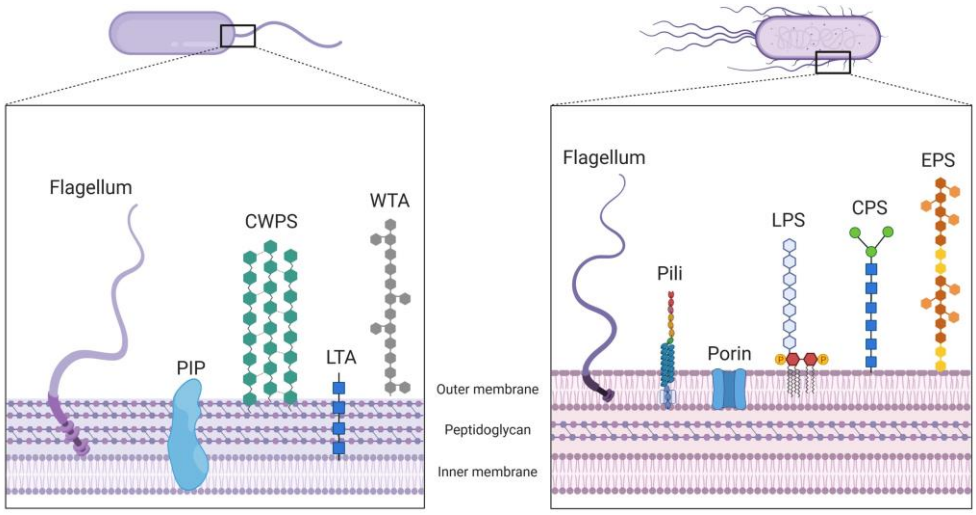


Fig. II.4 | Schematic illustrations of Gram-positive (left) and Gram-negative (right) cell surface-associated virulence factors that act as phage receptors. *PIP, phage infection protein; CWPS, cell wall polysaccharide; LTA, lipoteichoic acid; WTA, wall teichoic acid; LPS, lipopolysaccharide; CPS, capsular polysaccharide; EPS, exopolysaccharide*. Adapted from Shen and Loessner, 2021[103].

Phages performing such a lytic cycle have on occasion been misunderstood and conceptually opposed to phages performing a lysogenic cycle **(Fig.II.3)** - a cycle involving a defining step of viral genome integration in bacterial host genome - as if these mechanisms were mutually exclusive[75]. A bi-axis classification of phage cycles seems more fitting, in which the "lysis axis" on the one hand differentiates lytic phages (all phages whose intra-bacterial cycle is able to lead to bacterial lysis) from chronic phages, whereas the "lysogeny axis" on the other hand differentiate lysogenic phages (all phages whose intra-bacterial cycle may include a phase of viral genome integration to the host bacterial genome) from non-lysogenic phages[104]. This classification admittedly clarifies an extensive and often confusing glossary of ill-defined historical terms such as "virulent" or "temperate" phages. Four functional classes of phages are thus delineated **(Fig. II.5)**.

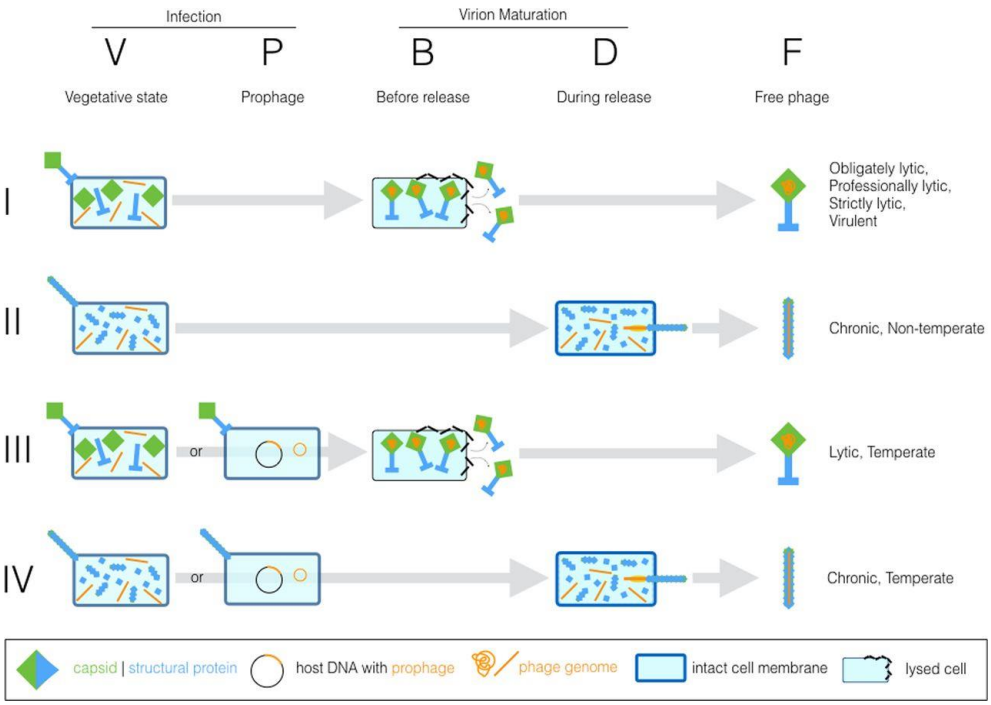


Fig. II.5 | Hobbs & Abedon distinction of phage states and cycles. From Hobbs & Abedon, 2016[104].

As PT canonically relies on a phage's lytic cycle to exert bactericidal therapeutic effect, only "type I" phages from this Hobbs & Abedon classification are considered for use in PT. As aforementioned, "type III" phages from this classification, to which belongs coliphage lambda **(Fig. II.3)**, are able to switch between lysogenic integrated prophage state and productive lytic cycle following complex metabolic cues and communication systems, some of which being the subject of recent discoveries[105, 106, 107]. Despite their ability to lead to bacterial lysis, however, and considering their tendency to propagate bacterial genes like antimicrobial resistance genes by transduction for example (see Section I.4), the use of such phages is actively avoided in practical PT: this is notably ensured by screening candidate phages for the absence of integrase or other integration-related genes during the genomic characterization phase[108].

II.3.3 Bacterial phage resistance

Bacterial phage resistance (BPR), the ability of a given bacterial isolate to acquire a resistant or less susceptible phenotype towards the bacteriolytic effect of a given phage, has been regularly observed from the early days of phage research in the early to mid-20th century. It has been cited as a major reason for its aforementioned demise during the following decades, based on concerns regarding its consequences on therapeutic efficacy[77]. Various works have however contradicted this intuition since then, notably

by proposing that the concomitant use of multiple phages would partly prevent this phenomenon[109, 110, 111]. The question of BPR impact on phage outcomes will be re-addressed in chapters III and V.

Mechanisms of BPR acquisition are numerous, and may vary along bacterial species. In 2019, for example. Azam and Tanji proposed a summarized classification of these mechanisms, highlighting that they could intervene at virtually any stage of the aforementioned lytic (or lysogenic) cycle **(Fig. II.6)**[109]. More recent works have since then delved into deeper in the study of many of these resistance mechanisms, and have unveiled the vertiginous complexity and variety of these intra-cellular structures and pathways, some of which remaining elusive to this date **(Fig. II.7)**[112, 113].

Phages can however dynamically develop numerous counter-resistance mechanisms, consistent with the permanent antagonistic co-evolution paradigm summarized by the "Red Queen Hypothesis"[114, 115]. We will not detail them further.

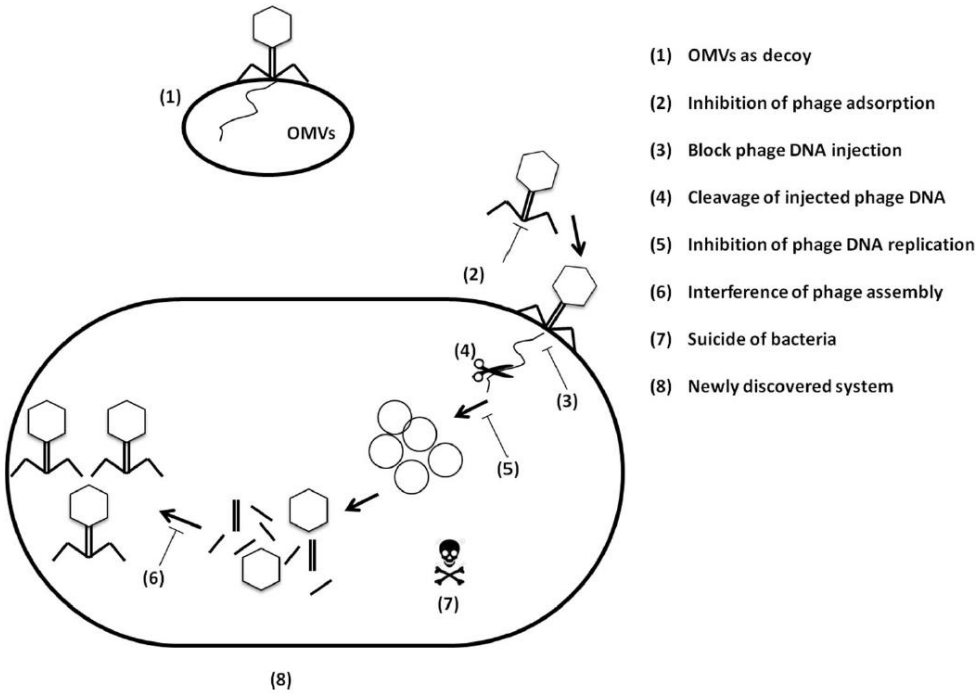
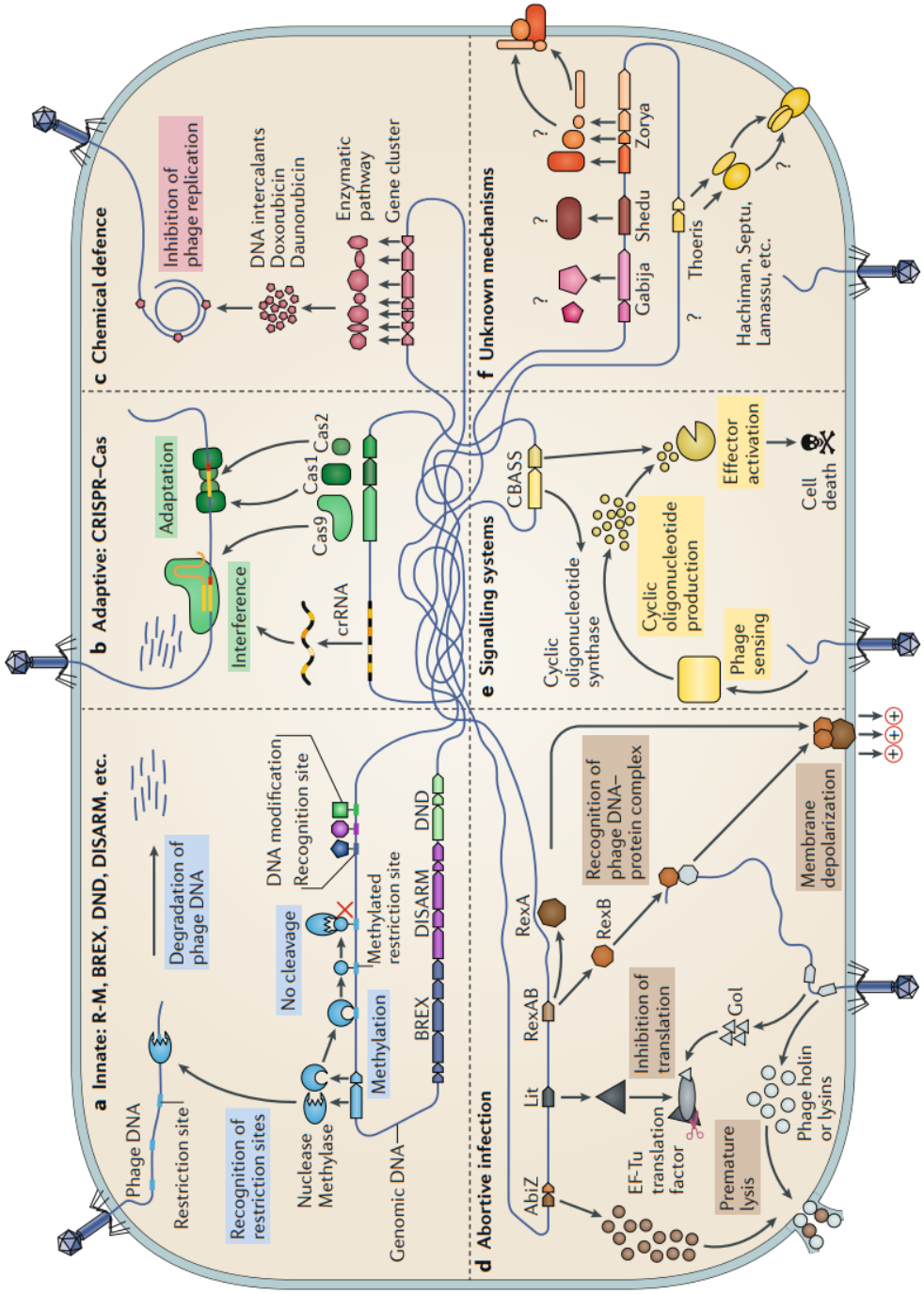


Fig. II.6 | Azam and Tanji's summary of phage resistance mechanisms. Numbers indicate the stage of phage infection at which the mechanism intervenes. *OMV, outer membrane vesicle.* From Azam and Tanji, 2019[109].


a Innate: R-M, BREX, DND, DISARM, etc.
Phage DNA
Restriction site
Recognition of restriction sites
Nuclease
Methylase
Methylation
Genomic DNA
No cleavage
Degradation of phage DNA
DNA modification
Recognition site
Methylated restriction site
BREX
DISARM
DND
b Adaptive: CRISPR–Cas
Adaptation
Interference
Cas1
Cas2
Cas9
crRNA
c Chemical defence
Inhibition of phage replication
DNA intercalants
Doxorubicin
Daunorubicin
Enzymatic pathway
Gene cluster
d Abortive infection
AbiZ
Lit
RexAB
RexA
RexB
Recognition of phage DNA–protein complex
Inhibition of translation
EF-Tu translation factor
Premature lysis
GoI
Phage holin or lysins
Membrane depolarization
e Signalling systems
CBASS
Cyclic oligonucleotide synthase
Cyclic oligonucleotide production
Phage sensing
Effector activation
Cell death
f Unknown mechanisms
Gabija
Shedu
Zorya
Thoeris
Hachiman, Septu, Lamassu, etc.

1293

Fig. II.7 | Antiviral defense systems in bacteria. *R-M, restriction-modification; CBASS, cyclic oligonucleotide-based anti-phage signaling system; Abi, abortive infection; BREX, bacteriophage exclusion; DISARM, defense islands system associated with R-M.* From Bernheim and Sorek, 2020[112].

II.4 *Therapeutic purposes* – phage therapy literature

II.4.1 Early days

Though d'Hérelle pioneered phage application towards PT and its early to mid-20th century bloom, as mentioned previously, the first published report of PT is attributed to others: Belgian medical doctor and phage researcher Richard Bruynoghe and his student Joseph Maisin, in 1921[116, 117].

Given the aforementioned historical context of PT's fate in the mid-to-late 20th century, however, it is unsurprising that a majority – though not the entirety - of the clinical literature available from that era would come from non-Western territories, i.e. ex-USSR countries for the most part.

Some publications, including some very recent ones, have extensively reviewed this vastly heterogenous 20th century literature. By favorably re-assessing PT's efficacy in these works along modern analytical standards, these works attempted to re-habilitate what were often colloquially

considered works of dubious rigor, a consideration based on unverified claims of absence of control groups for example[118, 119].

Nevertheless, since the beginning of the modern era of phage renaissance, considerable body of work has investigated PT-related outcomes in pre-clinical and clinical contexts, trying to abide by recent methodological standards.

II.4.2 Modern pre-clinical literature

Two recent extensive reviews have covered modern safety and efficacy trials of PT in animal models: a literature review by Melo and colleagues published in February 2020, and a systematic review with meta-analysis by Gomez-Ochoa and colleagues published in December 2022[120, 121].

The 2020 literature review by Melo and colleagues covered 60 distinct animal PT studies published between 2008 and 2019[120]. This review illustrated the experimental diversity of the field, with (number of covered studies in brackets, by decreasing order):

- Various types of clinical indications: bacteremia (13), lower respiratory tract infections (13), skin and soft tissue infections (12), gastrointestinal tract infections (9), eye and ear infections (4), upper respiratory tract infections (4), urinary tract infections (3), endocarditis (1) and dental infections (1)

- Various targeted pathogens (some studies investigating several concomitantly): *Pseudomonas aeruginosa (17), Staphylococcus aureus (11),*

Escherichia coli (10), Klebsiella pneumoniae (8), Acinetobacter baumanii (5), Enterococcus faecalis (3), Vibrio cholerae (2), Vibrio parahaemolyticus (2), Salmonella spp. (2), as well as single occurrences of *Mycobacterium ulcerans, Burkholderia cenocepacia*, and *Cronobacter turicensis*.

- Various administration routes, often consistent with the tested clinical indication (some studies investigating several concomitantly): intra-peritoneal (21), oral (10), intranasal (10), topical (10), intravenous (5), other localized injections including subcutaneous (5), intramuscular (3).

- Various animal models: mice representing the vast majority, completed by rarer occurrences of rats, rabbits, sheep, hamsters, pigs, dogs and minks.

- And above all various measured outcomes.

Melo and colleagues acknowledge that this variety in outcomes is an analytical challenge, and that other limitations arise from design choices such as the tendency to trigger infection by inoculating a normally lethal dose of bacteria, and initiating PT after an unrealistically short delay. They nevertheless draw a series of conclusions based on the analysis of these studies, such as the apparently undisputed safety profile of PT, regardless of administration route. Efficacy-related primary outcomes are also mostly met, but a series of efficacy criteria is identified. The most important one appears to be the choice of a relevant administration route with regard to the infection site, so that high loads of phages can be delivered at the direct contact of the targeted infection. According to clinical indications, other proposed criteria include the delivery of a sufficiently high titer of phages (though it is not

practically defined), via multiple administrations rather than a single one, and preferably using multiple phages at once rather than a single one.

In their 2022 meta-analysis, Gomez-Ochoa and colleagues address a larger body of work in a more systematic fashion regarding study selection and outcome analysis **(Fig. II.8)**[121].

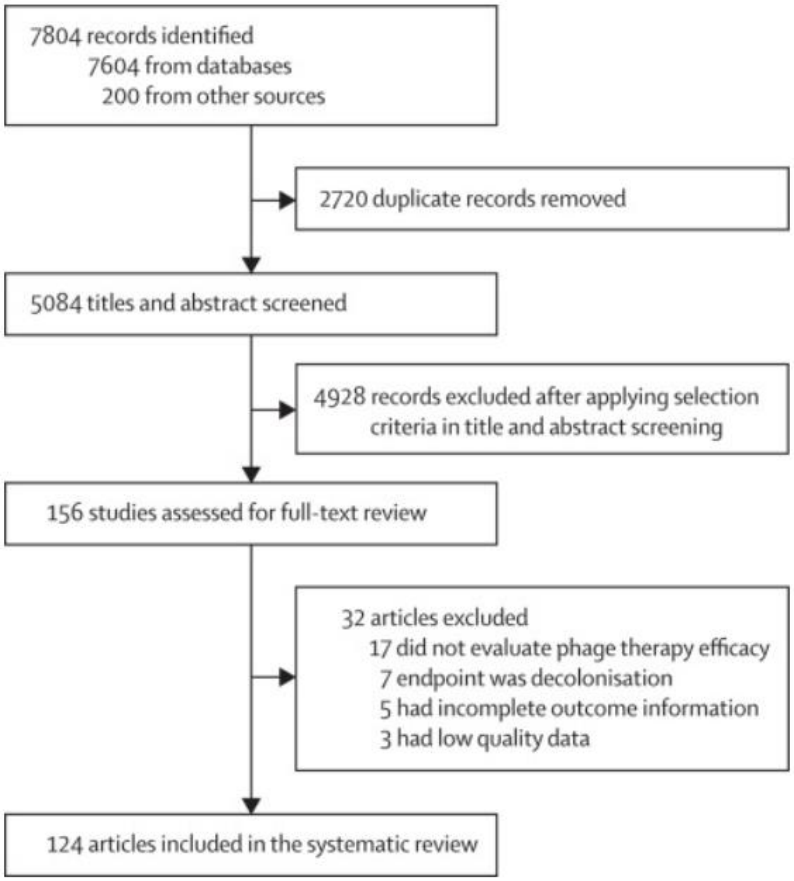


Fig. II.8 | Study selection algorithm for systematic review and meta-analysis of pre-clinical phage therapy studies. From Gomez-Ochoa et al., 2022[121].

The 124 articles selected by this method were then screened for bias risk assessment: a majority (70/124) of them appeared to be at high risk of bias, primarily due to the omission of information about the methodology (e.g. regarding randomization or blinding). Only the 54 remaining low-to-middle bias risk studies were kept for quantitative synthesis, which addressed both animal survival and tissue bacterial load outcomes.

Key strengths identified in all of these 54 studies was the systematic use of placebo control group, high animal sample sizes, and the absence of concomitant antibiotic treatment.

Overall, the results confirmed the general tendency for significant efficacy of PT in these models, with statistically significant meta-regression results regarding animal mortality in systemic infections, pneumonia and skin and soft tissue infection models, as well as a statistically significant reduction in tissue bacterial load in *S. aureus* skin and soft tissue infection models and *P. aeruginosa* pneumonia models.

The authors nevertheless warned that these results should be interpreted cautiously, given the occasional high heterogeneity between aggregated studies' sample sizes and a likely suboptimal estimation of publication bias. In addition, the results indicated that time-to-treatment delay typically represented a "best case scenario" for PT and likely influenced

results: the authors recommended ulterior pre-clinical studies would implement infection scenario more in line with what would be expected in human clinical trials.

II.4.3 Modern clinical literature

Modern clinical literature has similarly aimed to address the safety and efficacy of PT in various indications and administration routes. However, an important quantitative mismatch still prevails between exponentially abundant and often enthusiastic case-based literature (see Chapter III), and rarer clinical trials of more nuanced conclusions[119]. However, this asymmetry is progressively being resolved, and can be largely attributed to the regulatory and legal constraints mentioned in Section II.2.2.

Early phase I and phase I/II clinical trials of the modern phage era have nevertheless paved the way for the subsequent research enthusiasm. In 2009, Rhoads and colleagues carried and reported the first phase I safety trial of the era, declaring no safety concerns regarding the topical application of PT for the treatment of venous leg ulcers[122]. The same year, Wright and colleagues notoriously reported the first modern era phase I/II clinical trial to report not only favorable safety outcomes, but also favorable efficacy outcomes in the treatment of drug-resistant *Pseudomonas aeruginosa*-mediated chronic otitis externa by topical PT[123].

However, some subsequent clinical trials have generated less encouraging results. In a phase I/II trial published in 2016, Sarker and colleagues evaluated the safety and efficacy of anti-*E. coli* oral PT (using

either single T4 phage or a Russian multi-phage preparation) in the treatment of acute diarrhea in children from Bangladesh, compared to placebo[124]. Evidence of intact end-to-end travel of phages through the digestive tract was obtained, and still no safety concerns were raised. However, the trial failed to meet its efficacy-related outcomes of intra-intestinal phage titer amplification and improvement of diarrhea clinical outcomes. This failure was deemed multifactorial, attributed to insufficient spectrum coverage of the used coliphages, administration of insufficient phage titers, insufficient intra-intestinal titers of targeted *E. coli*, and only partial contribution of said *E. coli* to the treated diarrhea's pathogenesis.

In 2019, Jault and colleagues reported the results of a phase I/II clinical trial investigating the safety and efficacy of the 7-days treatment of burn wounds infections by the topical application of an anti-*P. aeruginosa* multi-phage cocktail compared to SOC management using 1% sulfadiazine silver emulsion cream[125]. Again, though no safety concerns were reported, the trial failed to illustrate the non-inferiority of topical PT at lowering bacterial burden in burn wounds compared to control. This was largely attributed to PT product shelf-life shortcomings, investigators being able to illustrate post-hoc that active phage titers in the therapeutic product had dramatically dropped between manufacturing and administration, reaching delivered concentrations as low as 10^2 plaque forming units(PFU)/mL instead of initially planned 10^6 PFU/mL.

In 2020, Leitner and colleagues published the results of a phase I/II trial investigating the safety and efficacy of intravesical PT based on a "Pyophage" cocktail in the pre-surgical treatment of complicated or recurrent

non-complicated urinary tract infection in adult men scheduled for transurethral resection of the prostate (TURP). Primary outcome was microbiological response to treatment at either day 7 or upon withdrawal from the trial. Two control groups were constituted in a 1:1:1 randomized fashion: SOC antibiotics or placebo. Again, no safety concerns were reported. Efficacy of PT turned out to be non-inferior to SOC antibiotic treatment, but surprisingly non-superior to placebo bladder irrigation. In their discussion, the authors mention a documented bacterial-reducing effect of the mechanical strain of placebo bladder irrigation which might have mitigated the differential effect compared to PT. In addition, the authors pointed out that the scarcity of similar literature caused a lack of guidelines to predict power and sample size calculations in such PT clinical trials. Lastly, they also mentioned suspicions of mismatch between *in vitro* activity of both antibiotics and phages during antimicrobial- and phage-susceptibility testing respectively, and *in vivo* effect, a bias whose contribution is complex to determine given the fact that this trial did not focus on a single bacterial species.

To conjointly analyze the results from both these contrasting case-based, series-based and trial-based literatures, recent works have performed their systematic review and meta-analysis. Uyttebroek and colleagues attempted this task in their 2022 publication, by systematically screening over 10 000 reports to eventually keep 52 studies (among which case reports, case series and clinical trials) for quantitative synthesis **(Fig. II.9)**[126].

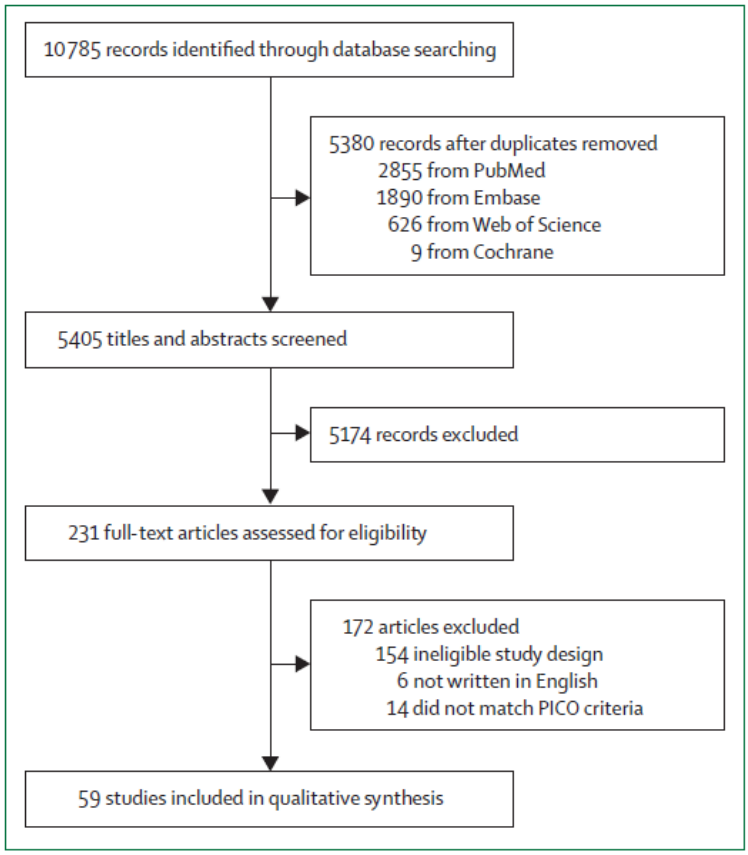


Fig. II.9 | Study selection algorithm for systematic review and meta-analysis of pre-clinical phage therapy studies. From Uyttebroek et al., 2022[126].

Quantifying their results in accordance to the GRADE (Grading of Recommendations Assessment, Development and Evaluation) approach, Uyttebroek and colleagues found out that adverse events had been reported in 7% (33/441) of phage-treated patients versus 15% (37/249) in control groups

patients when those existed, whereas clinical improvement was seen in 78.8% (1500/1904) of phage-treated patients versus 50% (17/34) in control groups, if any. Bacterial eradication was achieved in 86.7% (1266/1461) of phage-treated patients. However, most of the GRADE-derived evidence levels of these findings were generally classified as moderate for clinical trials, and as low or very low for case reports and case series. This was in part due to risk of indirectness (due to the occasional concomitant use of antibiotics) and/or other bias, such as publication bias. Another notable review published previously by El Haddad and colleagues in 2018 had reached similar results based on the systematic review of 30 studies specifically investigating the safety and efficacy of PT in the treatment of human infections du to MDR-ESKAPE pathogens[127].

Publication bias is a frequent criticism of such literature reviews, i.e. in this case the alleged tendency of researchers and/or journals to disproportionately publish cases where PT's efficacy seems obvious, at the detriment of the non-publication of cases where PT's efficacy appears disappointing. Such concerns are legitimate, but previous years have illustrated that there was significant publication potential and reading interest in cases reports challenging phage researcher's enthusiasm[128, 129, 130]. Nevertheless, additional prospective randomized controlled trials (RCTs) appear as the only solution to fully address this caveat.

In addition, the determination of clear analytical criteria to discriminate PT successes from failures in this literature appears as a major challenge. Consistent with some conclusions drawn by aforementioned pre-clinical works, Stacey and colleagues, in their 2022 systematic review, came

up with the following general criteria: "for efficacy to be observed, a therapeutic amount of the right phage(s) must be delivered to the right place to treat infections containing enough susceptible bacterial cells", claiming that all trials that did not demonstrate efficacy have failed to meet at least one of these criteria. This interesting conceptual "filter" still might not offer sufficient practical guidance in the definition of what exactly constitutes "the right place" or "enough susceptible bacterial cells", for example. This will be re-addressed in Chapter III.

At the time of writing, the Clinicaltrial.gov website (last access April 10th, 2024) lists 11 interventional PT-related clinical trials currently recruiting subjects: these investigate the safety and efficacy of PT in indications such as diabetic foot infections (2), cystic fibrosis-related or -unrelated pulmonary infections (2), urinary tract infections (2), prosthetic joint infections (1), surgical site infections (1), bacteremia (1), preterm infant necrotizing enterocolitis (1) and intractable constipation (1). The results of these trials might prove determining in shaping the future of PT clinical research and routine practice; they might also contribute to either dispel or reinforce the doubts and questions of the general medical community at a time where reasonable proof of PT's efficacy, specifically in such RCTs, is still mostly missing.

II.5 Afterword – Contextualization

Our personal thesis work, detailed in the next chapters, reflects a multi-axes approach to phage research based on an initial enthusiasm sparked in late 2017 by a single clinical case.

- **Chapter III** will start by detailing this peculiar, "founding" case and the multiple translational research approaches that have been explored on its basis. It will also mention clinical and translational research findings from other patients we were able to treat by PT from that point onwards.

- **Chapters IV and V** will then broaden the scope of our research to address the initial underlying problematic that contributed to the triggering of the dreadful infection in this initial patient: the infectious risk profile of (especially pediatric) liver transplantation recipients, and the contribution played by pre-surgical digestive asymptomatic carriage of drug-resistant bacteria in their pathogenesis.

- **Chapter IV** will detail the findings of a pilot clinical study that achieved the mono-centric screening of pediatric liver transplantation candidates.

- **Chapter V** will detail the results from the *in vitro* investigation of this digestive carriage problematic in an original experimental model: the Gut-On-A-Chip.

1547

1548

1549

1550

1551

1552

1553

1554

1555

1556

1557

1558

1559

1560

1561

1562

1563

Chapter III: Phage Therapy – Clinical cases and translational research

III.1 Bacteriophage-antibiotic combination therapy against extensively drug-resistant *Pseudomonas aeruginosa* infection to allow liver transplantation in a toddler

Brieuc Van Nieuwenhuyse[1]*, Dimitri Van der Linden[1,2], Olga Chatzis[2], Cédric Lood[3,4], Jeroen Wagemans[3], Rob Lavigne[3], Kaat Schroven[3], Jan Paeshuyse[5], Catherine de Magnée[6], Etienne Sokal[7], Xavier Stéphenne[7], Isabelle Scheers[7], Hector Rodriguez-Villalobos[8], Sarah Djebara[9], Maya Merabishvili[10], Patrick Soentjens[9,11], Jean-Paul Pirnay[10]

[1]Institute of Experimental and Clinical Research, Pediatric Department (IREC/PEDI), Université catholique de Louvain - UCLouvain, Brussels, Belgium. [2]Pediatric Infectious Diseases, General Pediatrics Department, Cliniques universitaires Saint-Luc, Université catholique de Louvain - UCLouvain, Brussels, Belgium. [3]Department of Biosystems, Laboratory of Gene Technology, KU Leuven, Leuven, Belgium. [4]Department of Microbial and Molecular Systems, Centre of Microbial and Plant Genetics, Laboratory of Computational Systems Biology, KU Leuven, Leuven, Belgium. [5]Department of Biosystems, Laboratory of Host Pathogen Interactions, KU Leuven, Leuven, Belgium [6]Pediatric and Transplantation Surgery, Cliniques universitaires Saint-Luc, Brussels, Belgium. [7]Pediatric Hepatology and Gastroenterology, Cliniques universitaires Saint-Luc, Brussels, Belgium. [8]Department of Microbiology, Cliniques universitaires Saint-Luc / Université catholique de Louvain - UCLouvain, Brussels, Belgium. [9]Center for Infectious Diseases, Queen Astrid Military Hospital, Brussels, Belgium. [10]Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium.[11]Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium.

Published in Nature Communications, 13, 5725 (2022)

III.1.1 Abstract

Post-operative bacterial infections are a leading cause of mortality and morbidity after ongoing liver transplantation. Bacteria causing these infections in the hospital setting can exhibit high degrees of resistance to multiple types of antibiotics, which leads to major therapeutic hurdles. Alternate ways of treating these antibiotic-resistant infections are thus urgently needed. Phage therapy is one of them and consists in using selected bacteriophage viruses as bactericidal agents in replacement or in combination with antibiotics. The use of phage therapy raises various research questions to further characterize what determines therapeutic success or failure. In this work, we report the story of a toddler who suffered from XDR *Pseudomonas aeruginosa* sepsis after liver transplantation. He was treated by a bacteriophage-antibiotic intravenous combination therapy for 86 days. This salvage therapy was well tolerated, without antibody-mediated phage neutralization. It was associated with objective clinical and microbiological improvement, eventually allowing for liver retransplantation, and complete resolution of all infections over a five-year follow-up. Clear *in vitro* phage-antibiotic synergies were observed. The occurrence of bacterial phage resistance did not result in therapeutic failure, possibly due to phage-induced virulence tradeoffs, which we investigated in different experimental models.

III.1.2 Introduction

Post-operative bacterial infections are a leading cause of mortality and morbidity after ongoing liver transplantation[131]. The spread and selection of antimicrobial resistance mechanisms among bacteria over the last decades now lays a considerable burden on global health, being directly responsible for more than a million yearly deaths[25]. These resistant bacteria have also become more frequently involved in the aforementioned post-operative infections[131]. *Pseudomonas aeruginosa* is a bacterial species responsible for a specifically high number of hospital-acquired bacterial infections, in part because of its ability to thrive in microbial "reservoirs" in the hospital setting[132]. *P. aeruginosa* can be considered a critical clinical pathogen for its numerous virulence factors, its ability to persist in a self-protective biofilm state, and for both its intrinsic and acquired mechanisms of multi-drug resistance[25, 133]. PT has regained interest as a complementary tool in the fight against multi-drug resistant bacterial infections[134], including those due to *P. aeruginosa*[135]. PT's efficacy and safety in the treatment of human infections through various administration routes are globally promising and have been the subject of a growing interest in recent clinical research[126].

In September 2018, a male toddler suffering from biliary atresia was transferred from Algeria to the Saint-Luc University Hospital (*Cliniques universitaires Saint-Luc - CUSL*). ABO-incompatible (ABOi) living-donor liver transplantation (LDLT, day 0) was performed using an adapted protocol,

including rituximab and plasmapheresis for optimal immunosuppression **(Fig. III.1)**.

At day 20 post-LDLT, routine active surveillance (rectal swab) showed nosocomial acquisition of *P. aeruginosa*. The isolated *P. aeruginosa* strain's antibiogram suggested an extensively drug-resistant (XDR) phenotype, with susceptibility to colistin only and intermediate susceptibility to aztreonam (data not shown). At day 53, the toddler presented intrahepatic biliary strictures, multiple hepatic abscesses, cholangitis and a severe septicemia caused by XDR *P. aeruginosa* **(Fig. III.1)**. Despite intravenous (IV) antibiotic therapy with gentamycin, colistin and high doses of aztreonam, blood and abscess samples continued to grow XDR *P. aeruginosa*. Concomitantly, the C-reactive protein (CRP) ranged from 89 to 189 mg/L. The child remained clinically septic and unstable and, for the fifth time since LDLT, had to be admitted to the PICU **(Fig. III.1)**.

1658

1659

4 PICU stays
between Day 0 – 20
LDLT
ABOi
Day 0
Microbiology 1
Escherichia coli
Bacteremia due to
digestive perforation
Day 5
Complication
Increasing rejection
Day 24
Microbiology 2
Undocumented sepsis
Day 15
Microbiology 3
XDR (VIM) Pseudomonas
aeruginosa
digestive tract carriage
Day 20
Microbiology 4
CMV infection
Day 24
PICU stay 5
XDR P. aeruginosa sepsis
Day 53 – 66
P. aeruginosa
isolates S1-S7
Day 58 – 66
Microbiology 5
Enterococcus faecium
bacteremia
Uncontrolled CMV infection
Day 42
Microbiology 6
XDR (VIM) P. aeruginosa
bacteremia
Uncontrolled CMV infection
Day 53
Microbiology 7
XDR (VIM) P. aeruginosa
bacteremia
Uncontrolled CMV infection
Day 58
EC approval
Day 57
Microbiology 8
Resurgent XDR (VIM) P.
aeruginosa bacteremia
Day 66
Microbiology 9
P. aeruginosa translocation
from liver abscesses to the
bloodstream
Days 84 and 87
PICU stay 6
Day 129 – 132
DDLT
ABOc
Day 129
Microbiology 10
E. faecium bacteremia
Stenotrophomonas maltophilia
fecal carriage
Day 127
D-10
D0
D10
D20
D30
D40
D50
D60
D70
D80
D90
D100
D110
D120
D130
D140
D150
26 September 2018 (D0)
3 February 2019 (D 129)
Pre ABOi LDLT
Rituximab, tacrolimus,
methylprednisolone
3 plasmapheresis
Day -14 – D0
Ceftriaxone,
metronidazole
Day 5 – 18
Vancomycin
Day 15 – 20
Ganciclovir
Day 24 – 43
Foscarnet
Day 43 – 54
Ganciclovir
Day 54 – 157
Vancomycin
Day 42 – 157

Fig. III.1 | Clinical timeline. Timeline of the most relevant surgical procedures (light green), microbiological results (light blue), antibiotic therapies (dark grey), pre- and post-liver transplantation protocols (blue-green), and phage therapy (magenta). The child developed several early-onset post-operative complications, including liver rejection, anaphylactic shock on rituximab, biliary digestive anastomosis perforation followed by an *Escherichia coli* and *Enterococcus faecium* sepsis, and cytomegalovirus (CMV) infection. At day 53 post-LDLT, he entered a severe septicemia due to XDR *Pseudomonas aeruginosa*. ABOc, ABO compatible; ABOi, ABO incompatible; BFC1, "bacteriofaagcocktail 1"; CMV, cytomegalovirus; DDLT, deceased-donor liver transplantation; EC, ethics committee; IA, intra-abdominal; IL, intralesional; IV, intravenous; LDLT, living-donor liver transplantation; pfu, plaque forming unit; PICU, pediatric intensive care unit; VIM, Verona integron-encoded metallo-β-lactamase; XDR, extensively drug-resistant.

The ongoing systemic infection was a contraindication for retransplantation. Because the antibiotic therapy failed and the child's situation was critical, the decision was taken at day 58 post-LDLT to drain the hepatic abscesses, and to initiate PT under the umbrella of article 37 (unmet medical need) of the Declaration of Helsinki and after Ethics Committee approval **(Fig. III.1)**. BFC1 ("bacteriofaagcocktail 1") is a phage cocktail produced by the Queen Astrid Military Hospital (QAMH) in Brussels, containing one *Staphylococcus aureus* phage (ISP) and two *P. aeruginosa* phages (PNM and 14-1)[136]. Before clinical application, the phage preparation was analyzed and approved by the Belgian Institute for Public Health (Sciensano), according to the quality criteria set in the general monograph (v1.0) for the production of phage pharmaceutical ingredients[91]. All three phages composing BFC1 have been sequenced and fully characterized. None of these phages contain any known genetic determinants coding for lysogeny, toxins, or antibiotic resistance. All three phages are considered strictly lytic. Based on the genome analysis, Sciensano issued a "green" genomic passport, certifying the absence of any known genetic determinants that would make these phages unsuitable for therapeutic use. BFC1 was purified using EndoTrap® affinity matrix and then certified by Sciensano to contain less than 5 EU/mL of endotoxins.

Ten milliliters (1 mL/kg) of BFC1 [10^7 plaque forming units (pfu)/mL] were administered by a daily six-hour IV infusion. Daily intralesional (IL) injections of 1 mL BFC1 were performed through the patient's biliary drainage catheter, but were suspended after seven days, because they caused abdominal discomfort in the child. Thirty-six hours after PT initiation, two subsequent blood cultures were negative. However, between days 4 and 8

after initiation of PT, blood and abscess pus cultures revealed the presence of *P. aeruginosa*. At day 8 (day 66 post-LDLT), the IV phage dose was doubled (2 mL/kg) and this for the remaining duration of the treatment. This led to the durable eradication of *P. aeruginosa* from the bloodstream; although two transient *P. aeruginosa* translocations from liver abscesses to the bloodstream were observed on day 27 and day 30 after PT initiation, they both disappeared in less than 24 hours without new therapeutic measures, and had no impact on the child's stable clinical state.

PT combined with antibiotics controlled the *P. aeruginosa* bloodstream infection, but did not eradicate *P. aeruginosa* in the hepatic lesions. The child remained clinically stable outside of the PICU with intermittent fever and persistent inflammation. This stable state was maintained until a postmortem ABO compatible (ABOc) liver was available for retransplantation, after 72 days of IV PT. Two days prior to retransplantation, the child developed a bacteremia with *Enterococcus faecium*, which was successfully treated with vancomycin. *Stenotrophomonas maltophilia* fecal carriage was also newly identified.

During the deceased-donor liver transplantation (DDLT), 250 mL of BFC1 was used to rinse the abdominal cavity **(Fig. III.1 and Fig. III.2)**. This procedure was well tolerated and IV PT was continued for two more weeks. Not surprisingly, culture of tissue samples from the removed diseased liver revealed the presence of *P. aeruginosa*, *E. faecium*, and *S. maltophilia*. The child was also treated with a wide combination of antibiotics (cotrimoxazole, vancomycin, colistin, ganciclovir, fluconazole, aztreonam and gentamycin) for 28 days post-DDLT **(Fig. III.1)**. In total, the child received 86

uninterrupted days of IV PT (72 days pre- and 14 days post-DDLT) **(Fig. III.1)**. No adverse event related to the IV PT was observed during hospitalization.

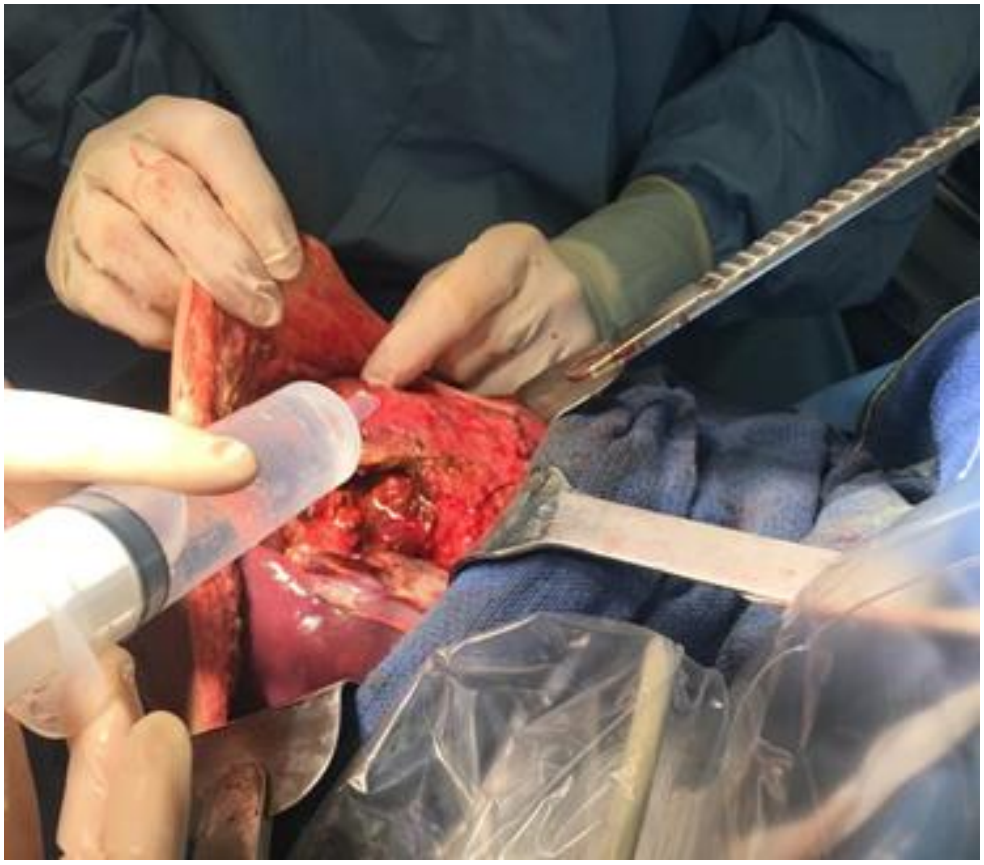


Fig III.2 | Intraoperative intra-abdominal phage therapy. Instillation of 250 mL of BFC1, containing 10_7 plaque forming units (PFU)/mL of phages ISP, PNM and 14-1, for 30 minutes, during the anhepatic phase of deceased-donor liver transplantation.

Five years after this second liver transplantation, the child is doing fine on immunosuppressive medication, with normal liver function, no transplant rejection and no further infectious outbreaks. Total clearance of *P. aeruginosa* colonization was observed.

In this work, we investigate the net contribution of PT in the child's favorable outcome through three phenomena that shed light on the *in vivo* anti-bacterial action of phages, in view of future salvage therapies and clinical trials: (i) in vivo bacterial phage resistance (BPR) emergence and possible incurred phage-induced virulence tradeoffs (PIVT), (ii) phage-antibiotic synergy (PAS), and (iii) phage immune neutralization (PIN).

III.1.3 Results and Discussion

Seven XDR *P. aeruginosa* isolates, retrieved from four blood cultures ($Pa1_{BS}$, $Pa2_{BR}$, $Pa4_{BR}$, and $Pa7_{BS}$) and three hepatic abscess pus cultures ($Pa3_{LS}$, $Pa5_{LR}$, and $Pa6_{LR}$) were retained for further analysis **(Table III.1)**. These isolates were sampled during what was arguably the most critical period in the history of this case, from day 58 to day 66 post-LDLT. Only the first one ($Pa1_{BS}$) was obtained before (4 hours) PT initiation, whereas the other six ($Pa2_{BR}$-$Pa7_{BS}$) were retrieved during the first week of PT. Due to the sense of urgency brought on by XDR *P. aeruginosa* bacteremia, the susceptibility of the *P. aeruginosa* isolates to the phages of the BFC1 cocktail could not be assessed prior to the start of PT. Two days later, we observed that only one of the three phages that make up cocktail BFC1, *P. aeruginosa* phage PNM, showed confluent lytic activity on pre-PT *P. aeruginosa* blood

isolate $Pa1_{BS}$ and subsequent isolates $Pa3_{LS}$ and $Pa7_{BS}$. Four of these seven isolates were phenotypically resistant to all phages present in BFC1: $Pa2_{BR}$, $Pa4_{BR}$, $Pa5_{LR}$, and $Pa6_{LR}$ **(Table III.1)**.

Name	Isolation site	Isolation date	Time relative to PT start	Phage lytic activity		
				PNM (10^9 pfu/mL)	PNM (10^7 pfu/mL)	PNM EOP
$Pa1_{BS}$	Blood	23 Nov 18	- 4 hours	++++	++	0.3
$Pa2_{BR}$	Blood	24 Nov 18	+ 8 hours	-	-	-
$Pa3_{LS}$	Liver	24 Nov 18	+ 11 hours	++++	+++	0.2
$Pa4_{BR}$	Blood	27 Nov 18	+ 4 days	-	-	-
$Pa5_{LR}$	Liver	27 Nov 18	+ 4 days	-	-	-
$Pa6_{LR}$	Liver	27 Nov 18	+ 4 days	-	-	-
$Pa7_{BS}$	Blood	1 Dec 18	+ 8 days	++++	+++	0.27

EOP, efficiency of plating; pfu, plaque forming unit; ++++, confluent lysis; +++, semi-confluent lysis; ++, opaque lysis; -, no lysis

Table III.1 | Phage susceptibility of seven *Pseudomonas aeruginosa* isolates retrieved before ($Pa1_{BS}$) and during ($Pa2_{BR}$-$Pa7_{BS}$) phage therapy. The nomenclature of these isolates consists of: [Pa] for *P. aeruginosa*; [number] for chronological order of isolation; [first letter: B/L] for origin of isolate, either from Blood culture or Liver abscess pus culture; [second letter: S/R] for phenotypic Susceptibility or Resistance of the isolate to phage PNM. *P. aeruginosa* isolates $Pa1_{BS}$, $Pa2_{BR}$, $Pa4_{BR}$, and $Pa7_{BS}$ were retrieved from blood samples harvested on days 57, 58, 61, and 65 post-LDLT, respectively. *P. aeruginosa* isolates $Pa3_{LS}$, $Pa5_{LR}$, and $Pa6_{LR}$ were isolated from liver abscess pus on days 58 ($Pa3_{LS}$) and 61 ($Pa5_{LR}$ and $Pa6_{LR}$). Phage susceptibility was determined by spot test.

III.1.3.1 Antimicrobial Resistance, Phage Resistance, Induced Tradeoffs

Whole genome sequencing (WGS) of the seven *P. aeruginosa* isolates considered was performed combining long-read and short-read sequencing technologies[137]. WGS and subsequent Single Nucleotide Polymorphism (SNP) analysis confirmed that isolates $Pa1_{BS}$-$Pa7_{BS}$ all belong to the same *P. aeruginosa* strain with over 99.9999% ANIb similarity and a maximum distance of 3 SNPs. *In silico* analysis revealed that the strain belongs to sequence type ST111 (serotype O12), known for its prevalence in nosocomial *P. aeruginosa* infections and its extensively-drug resistant profile[138]. Five of the isolates harbor the bla_{VIM-2} gene coding for a VIM (Verona Integron-encoded Metallo-beta-lactamase) type carbapenemase, which is a common feature of this high-risk clone[138]. WGS revealed that each isolate contained six to eight genetic determinants known to confer antimicrobial resistance **(Fig. III.3)**. The distribution of these genetic determinants among the isolates does not indicate that any of them would have been selected by the administration of antibiotics to the patient. The circular chromosomic views (CCV) of the bacterial genomes are depicted in **Figure III.4**. Additionally, WGS analysis showed that the four isolates expressing BPR possess genetic alterations in the 5.8-5.9 Megabases (Mb) chromosomic region, which affect the development of the Type IV pili (T4P) complex. This region contains various T4P-related genes such as *pilM*, *pilO2*, *pilP*, and *pilV*[139]. In isolate $Pa6_{LR}$, T4P variation consisted of an SNP in the *pilB* locus, whereas in the three other phage-resistant isolates ($Pa2_{BR}$, $Pa4_{BR}$ and $Pa5_{LR}$), it consisted in a regional deletion in a putative *P. aeruginosa* Genomic Island (PAGI). In

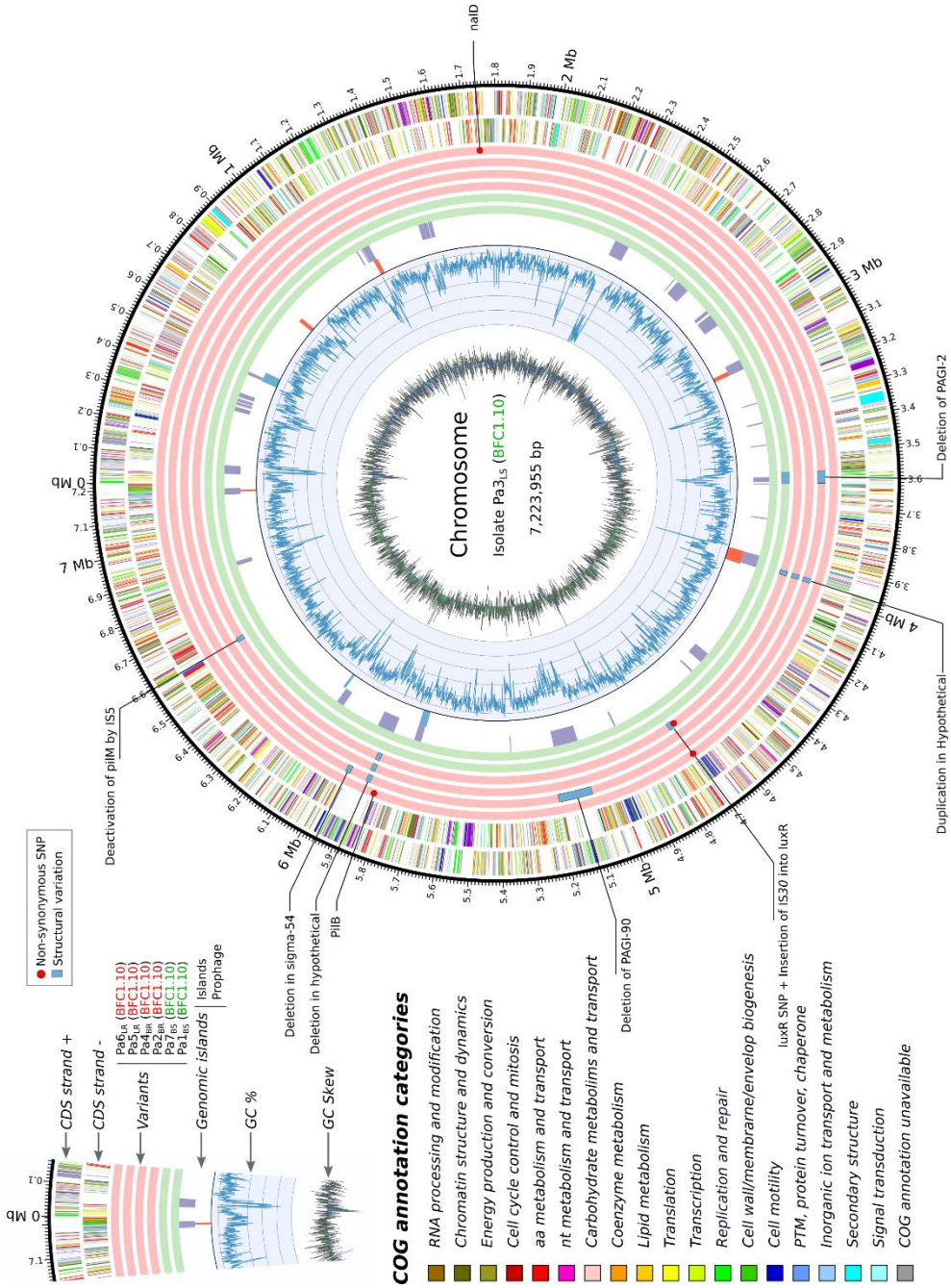
addition, alternative or complementary ways of achieving T4P-modification can be suspected, for example *pilM* deactivation by IS5 transposase activity in isolate $Pa2_{BR}$, or a PAGI-90 deletion in isolate $Pa5_{LR}$. Phage PNM was shown to require the presence T4P for successful infection[140]. Acquiring bacterial phage resistance (BPR) through genetic alteration of outer phage adsorption determinants is one of the most frequently described mechanisms of BPR acquisition[109, 141]. It is thus possible that a component of the T4P structure serves as a specific adsorption site for phage PNM.

a
Resistant
Intermediate
Susceptible
Not tested
Pa1BS
Pa2BR
Pa3LS
Pa4BR
Pa5LR
Pa6LR
Pa7BS
QAMH-MIC
STLUC-disk
STLUC-MIC
Amikacin
Gentamicin
Tobramycin
Aminoglycosides
Ticracillin/clav
Piperacillin
Piperacillin/tazobactam
Ticracillin
Beta-lactams
Ceftazidim
Cefepim
Cephalosporines
Aztreonam
Monobactams
Imipenem
Meropenem
Carbapenems
Levofloxacin
Ciprofloxacin
Fluoroquinolones
Colistin
Polymixins
Trimethroprim/sulfamethoxazole
Sulfonamide

b

Isolate	Number of AMR genes	Aminoglycoside : **aac(6')-29a**	Aminoglycoside : **aac(6')-29b**	Kanamycin : **aph(3')-IIb**	Beta-lactam : **blaOXA-395**	Cephalosporin : **blaPDC-55**	Carbapenem : **blaVIM-2**	Chloramphenicol : **catB7**	Fluoroquinolone : **crpP**	Fosfomycin : **fosA-354827590**	Sulfonamide : **sul1**
Pa1$_{BS}$	6	-	-	+	+	+	-	+	+	+	-
Pa2$_{BR}$	9	-	+	+	+	+	+	+	+	+	+
Pa3$_{LS}$	10	+	+	+	+	+	+	+	+	+	+
Pa4$_{BR}$	10	+	+	+	+	+	+	+	+	+	+
Pa5$_{LR}$	6	-	-	+	+	+	-	+	+	+	-
Pa6$_{LR}$	8	+	-	+	+	+	+	+	+	+	-
Pa7$_{BS}$	8	+	-	+	+	+	+	+	+	+	-

Fig. III.3 | Antibiotic susceptibility phenotype (a) and related genes (b) of seven *Pseudomonas aeruginosa* isolates retrieved before (Pa1$_{BS}$) and during (Pa2$_{BR}$-Pa7$_{BS}$) phage therapy. The nomenclature of these isolates consists of: [Pa] for *P. aeruginosa*; [number] for chronological order of isolation; [first letter: B/L] for origin of isolate, either from Blood culture or Liver abscess pus culture; [second letter: S/R] for phenotypic Susceptibility or Resistance of the isolate to phage PNM. *P. aeruginosa* isolates Pa1$_{BS}$, Pa2$_{BR}$, Pa4$_{BR}$, and Pa7$_{BS}$ were retrieved from blood samples harvested on days 57, 58, 61, and 65 post-LDLT, respectively. *P. aeruginosa* isolates Pa3$_{LS}$, Pa5$_{LR}$, and Pa6$_{LR}$ were isolated from liver abscess pus on days 58 (Pa3$_{LS}$) and 61 (Pa5$_{LR}$ and Pa6$_{LR}$). Minimum Inhibitory Concentrations were determined at the Queen Astrid Military Hospital (QAMH-MIC) using VITEK 2 technology and the ADVANCED EXPERT SYSTEM (both bioMérieux Benelux, Brussels, Belgium), and were determined at Saint-Luc University Hospital (StLuc-MIC) through automated microdilutions (Phoenix, Becton Dickinson, Franklin Lakes, NJ, USA). Antibiotic susceptibility was also assessed through disk diffusion method in Saint-Luc University Hospital (StLuc-disk) using Adagio reading technology (Bio-Rad, Marnes-la-Coquette, France). QAMH-MIC, minimum inhibitory concentrations determined at the Queen Astrid Military Hospital ;StLuc-MIC, minimum inhibitory concentrations determined at Saint-Luc University Hospital ; StLuc-disk, antibiotic susceptibility determined at Saint-Luc University Hospital by disk diffusion method, AMR, antimicrobial resistance

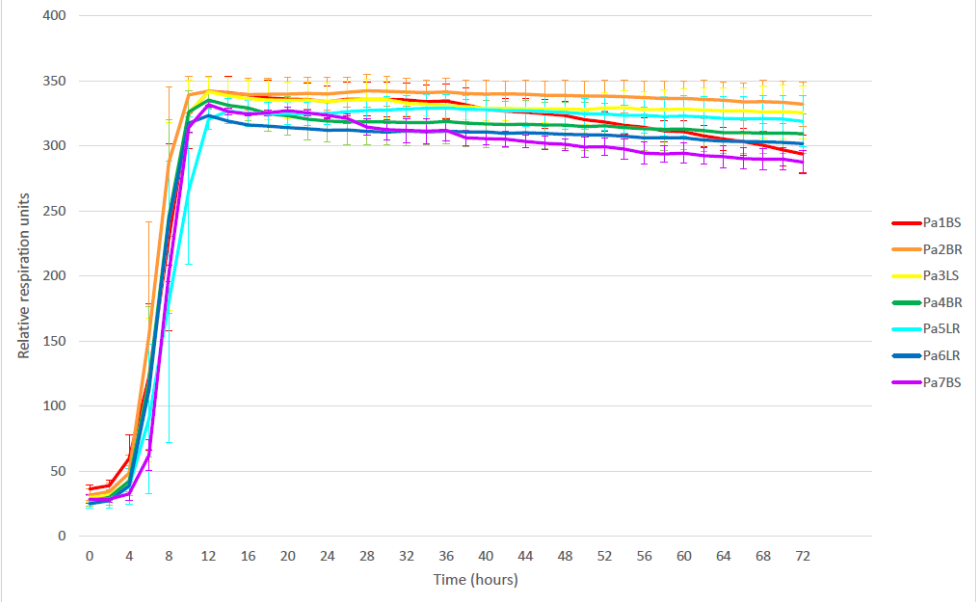

Chromosome
Isolate Pa3LS (BFC1.10)
7,223,955 bp
naID
Deletion of PAGI-2
Duplication in Hypothetical
luxR SNP + Insertion of IS30 into luxR
Deletion of PAGI-90
PilB
Deletion in hypothetical
Deletion in sigma-54
Deactivation of pilM by ISS
Non-synonymous SNP
Structural variation
CDS strand +
CDS strand -
Variants
Pa6LR (BFC1.10)
Pa5LR (BFC1.10)
Pa4BR (BFC1.10)
Pa2BR (BFC1.10)
Pa7BS (BFC1.10)
Pa1BS (BFC1.10)
Genomic islands
Islands
Prophage
GC %
GC Skew
COG annotation categories
RNA processing and modification
Chromatin structure and dynamics
Energy production and conversion
Cell cycle control and mitosis
aa metabolism and transport
nt metabolism and transport
Carbohydrate metabolims and transport
Coenzyme metabolism
Lipid metabolism
Translation
Transcription
Replication and repair
Cell wall/membrarne/envelop biogenesis
Cell motility
PTM, protein turnover, chaperone
Inorganic ion transport and metabolism
Secondary structure
Signal transduction
COG annotation unavailable

1801

Fig. III.4 | Circular chromosomic view (CCV) of the bacterial genomes of seven *Pseudomonas aeruginosa* isolates retrieved just before ($Pa1_{BS}$) and during ($Pa2_{BR}$-$Pa7_{BS}$) phage therapy. The CCV algorithm chose isolate $Pa3_{LS}$ as reference (inner circle). From the inner to outer rings of the CCV, two green rings display the genomic variations in phage PNM-susceptible isolates $Pa1_{BS}$ and $Pa7_{BS}$, and four outer red rings display the genomic variations in phage PNM-resistant isolates $Pa2_{BR}$, $Pa4_{BR}$, $Pa5_{LR}$, and $Pa6_{LR}$. The two multi-colored outer rings display the protein annotations (categories) as present in the database of Clusters of Orthologous Groups of proteins (COGs). aa, amino acid; bp, basepairs; CDS, coding sequence; IS, insertion sequence; Mb, megabases; nt, nucleotide; PAGI, *Pseudomonas aeruginosa* genomic island; PTM, post-translational modification; SNP, single-nucleotide polymorphism

The retrieval of blood-circulating *P. aeruginosa* isolates, which show *in vitro* insensitivity to all administered antibiotics and phages that were administered ($Pa2_{BR}$ and $Pa4_{BR}$), could have been considered as a sign of impending therapeutic failure. Yet, PT initiation led to the immediate and sustained improvement of the child's clinical state, and eventually to the permanent exclusion of *P. aeruginosa* from the bloodstream. It has often been suggested that besides their primary lytic effect, phages can also indirectly threaten bacterial prosperity by exerting a deteriorating selective pressure. BPR can come with a fitness cost, as it can require genetic variations which can lead to the loss of key survival elements, like intrinsic virulence or antimicrobial resistance[142, 143]. Facing such fitness costs, bacteria are "caught

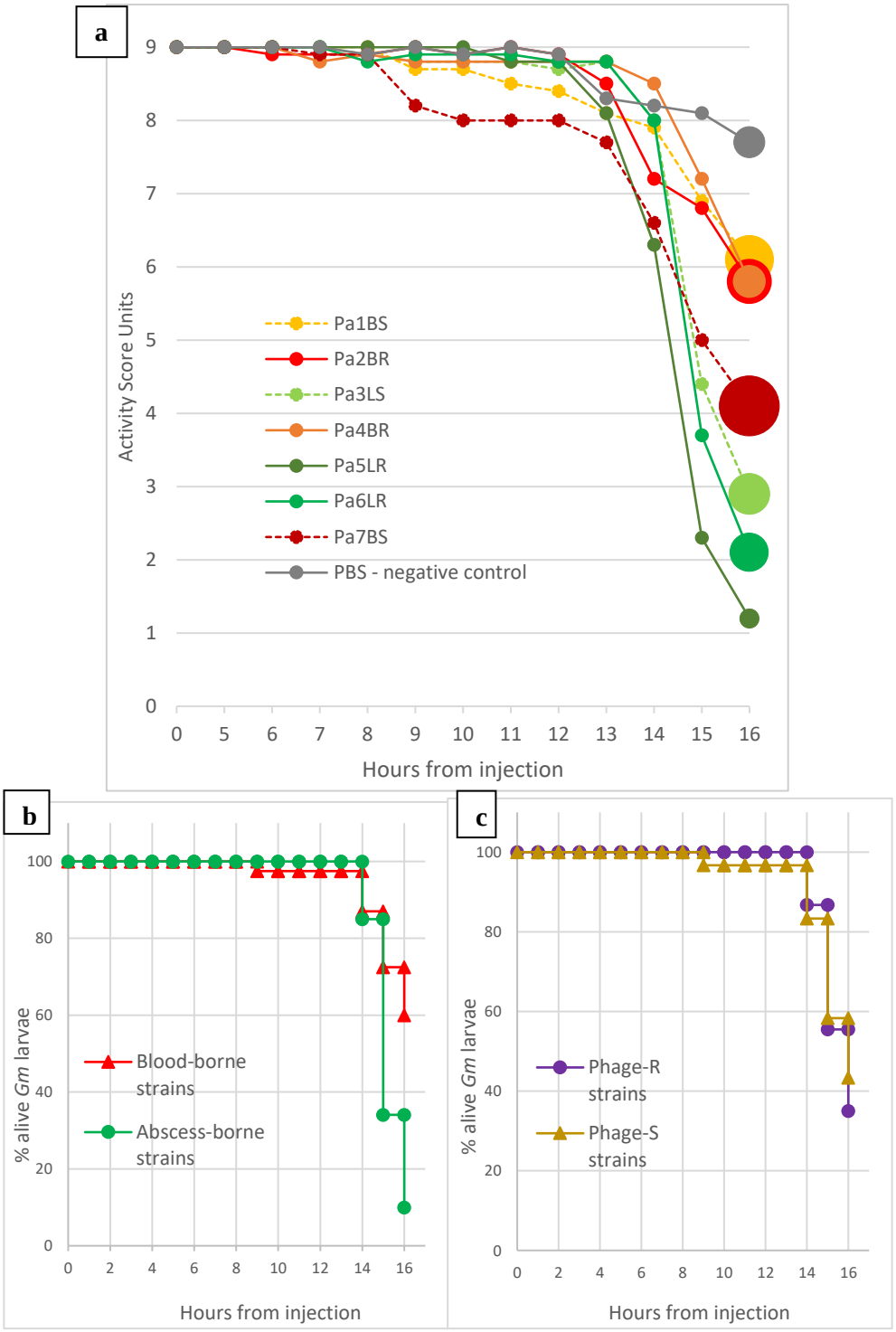
in a vice" where escaping phage lysis results in recovering previous antimicrobial susceptibility and/or losing intrinsic virulence. Since T4P regulates virulence in *P. aeruginosa*,[144] the occurrence of phage-induced virulence tradeoffs (PIVT) was plausible. All seven isolates were analyzed using an OmniLog® system in identical conditions, which did not highlight any significant difference in bacterial growth rate between the isolates over 72 hours **(Fig. III.5)**. Therefore, we conducted *Galleria mellonella* (*Gm*) virulence assays **(Fig. III.6.a-c)**. Batches of *Gm* larvae were inoculated with any of the seven retained *P. aeruginosa* isolates (or with a phosphate-buffered saline control), and their viability (activity score) was monitored hourly to look for possible differences in virulence between the different isolates **(Fig. III.7)**. In addition, a second independent assay was performed to assess the virulence of the isolates. We performed a cell viability assay using HeLa cells. These human cells were incubated together with the *P. aeruginosa* isolates for 24 hours, after which HeLa cell viability was assessed **(Fig. III.6.d)**.



1842

Fig. III.5 | Growth kinetics of the 7 *P. aeruginosa* isolates. Growth kinetics were measured using an OmniLog® system. Bacterial proliferation is presented through relative units of cellular respiration. Results represent mean values of three experiments (biological replicates) with error bars representing the standard deviations of the means.

1843


a
Activity Score Units
Hours from injection
Pa1BS
Pa2BR
Pa3LS
Pa4BR
Pa5LR
Pa6LR
Pa7BS
PBS - negative control
b
% alive Gm larvae
Hours from injection
Blood-borne strains
Abscess-borne strains
c
% alive Gm larvae
Hours from injection
Phage-R strains
Phage-S strains

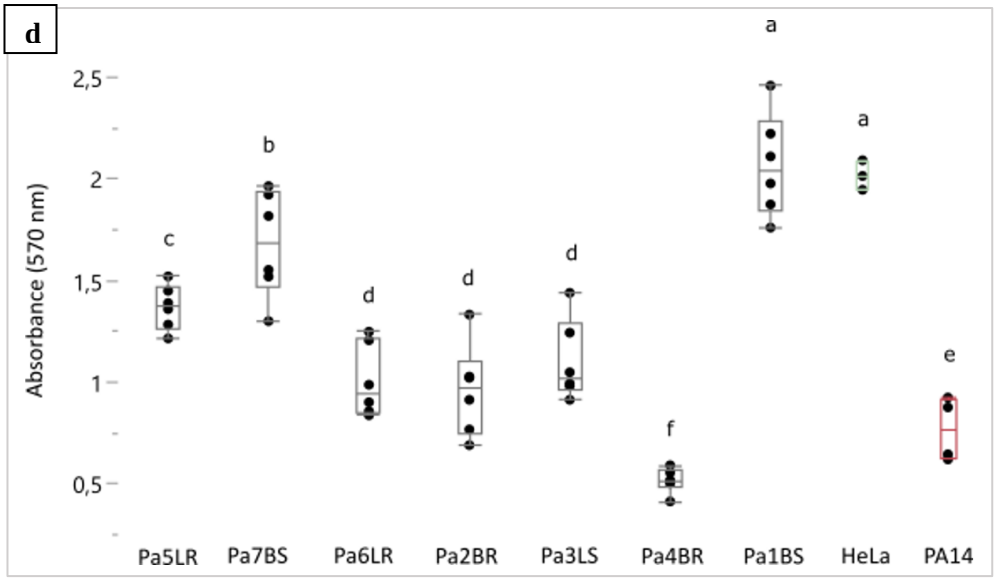


Fig. III.6 | Virulence assays. a Chronological evolution of the mean activity score of *Gm* larvae injected with phosphate-buffered saline (control) or any of the seven *Pseudomonas aeruginosa* isolates retrieved just before ($Pa1_{BS}$) and during ($Pa2_{BR}$-$Pa7_{BS}$) phage therapy. Full lines represent the mean activity scores of *Gm* larvae injected with any of four PNM-resistant *P. aeruginosa* isolates ($Pa2_{BR}$ and $Pa4_{BR}$-$Pa6_{LR}$) or phosphate-buffered saline (PBS). Dashed lines were used for the three PNM-susceptible isolates ($Pa1_{BS}$, $Pa3_{LS}$, and $Pa7_{BS}$). Note that first shown measurement after injection starts at +5 hours. Real scale standard deviations were not visually implementable, but the dot width of each final mean measurement correlates with its standard deviation. n = 10 biologically independent animals. **b** Log-rank test finds the three liver abscess-borne isolates ($Pa3_{LS}$, $Pa5_{LR}$ and $Pa6_{LR}$) significantly more virulent than the bloodborne isolates ($Pa1_{BS}$, $Pa2_{BR}$, $Pa4_{BR}$ and $Pa7_{BS}$): p-value = 0.0011. **c** Conversely, Log-rank test shows no significant difference in virulence when comparing the three phage-susceptible isolates […]

[…] ($Pa1_{BS}$, $Pa3_{LS}$, $Pa7_{BS}$) with the four phage-resistant isolates ($Pa2_{BR}$, $Pa4_{BR}$, $Pa5_{LR}$, $Pa6_{LR}$): p-value = 0.7552. **d** The absorbance at 570 nm was measured to evaluate the viability of HeLa cells. The highly virulent *P. aeruginosa* strain PA14 was used as a positive control whereas HeLa cells without the addition of a *P. aeruginosa* culture served as a negative control. A connected letter report was created to pairwise compare all isolates and controls (two-sided Student's t-test, p-value = 0.05, n = 6 biologically independent samples, no adjustment made for multiple comparisons). $Pa1_{BS}$ was found to not significantly reduce the viability of the cells, compared to the negative control. Box-plots elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range; points, individual values.

a

Category	Description	Score
Activity	No activity despite stimulation	0
	Minimal activity upon stimulation	1
	Normal activity upon stimulation	2
	Spontaneously active without stimulation	3
Melanization	Complete melanization : black larvae	0
	Brown larvae with darker spots	1
	≥3 spots on beige larvae	2
	<3 spots on beige larvae	3
	No melanization : cream white larvae	4
Survival	Dead	0
	Alive	2
Total	Sum of all categories, 0 to 9	

Figure III.7 | *Galleria mellonella* assay a An activity score was used to assess the *Galleria mellonella* (Gm) larvae health status after inoculation with each *Pseudomonas aeruginosa* isolate by assigning scores according to three major observation categories: larvae activity, melanization and survival. **b** Healthy larvae are cream-colored, have noticeable tonus and are spontaneously mobile. **c** as infection progresses, mobility weakens and color darkens to varying shades of beige and brown, with noticeable inter-individual variability highlighting the need to use batches of several larvae. **d** at some point in time, all larvae die then reach a total activity score of 0 upon complete melanization (black color).

No correlation was found between BPR-phenotype and prolonged survival of the larvae or virulence towards HeLa cells **(Fig. III.6.a, c, d)**. Moreover, no correlation was observed between the data of the two virulence assays, although both assessments revealed $Pa1_{BS}$ as the least virulent isolate. However, we consider that this does not necessarily rule out the occurrence of PIVT in the child. Indeed, results from our assay potentially suffer from survivor bias, since they can only be based on the phage-resistant isolates that we managed to retrieve and culture from the child's biological samples. Such isolates are the ones that managed to escape both phage lysis and immune system clearance, but their bioburden could ultimately be too low to sustain or re-establish proper infection in the child. Such isolates could be considered "remnant" colonizers, fallaciously representing a bacterial population that was largely decimated by a combination of phage lysis and PIVT.

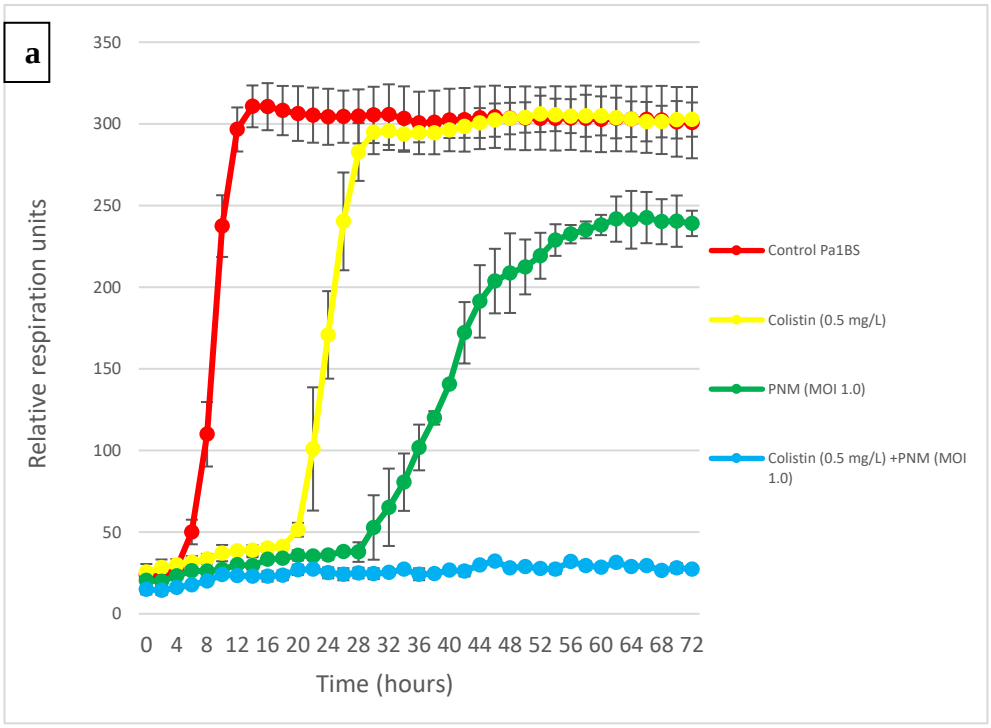
Based on the *Gm* assay, it should be noted that three isolates appeared significantly more virulent ($Pa3_{LS}$, $Pa5_{LR}$ and $Pa6_{LR}$) (p-value = 0.0011) **(Fig. III.6.b)**. According the to our WGS analysis, these isolates do not seem to share chromosomic characteristics that none of the other four isolates ($Pa1_{BS}$, $Pa2_{BR}$, $Pa4_{BR}$, and $Pa7_{BS}$) harbor. This does not rule out the possibility of a significantly higher virulence in the first three isolates, since the spontaneous generation of phenotypic, non-genetically-mediated heterogenicity regarding such characteristics within a clonal bacterial population is a known phenomenon[145]. However, we noticed that these three isolates were the ones retrieved from liver abscess pus, whereas the other less virulent isolates were bloodborne. While this correlation between liver origin and virulence of the isolate in *Gm* could be coincidental, it could also provide a base for another putative explanation of PIVT in this model. Indeed, PT's scheme of administration in our case was mainly intravenous. This makes it safe to assume that bacteria in the bloodstream were significantly more exposed to phages than bacteria in the liver abscesses. The lower virulence in *Gm* of the bloodborne "bacterial subpopulation" then correlates with its higher exposition to phages, whereas the lesser exposed liver subpopulation exhibits higher virulence. Under this paradigm, PIVT could been seen as a "subpopulation-scale" mechanism, where correlation between intensity of exposition to phages and virulence loss are not dependent on the phage-susceptibility of some specific bacterial individuals in these subpopulations. It should be noted, however, that the results of the subsequent HeLa cell assay did not reproduce this clustering.

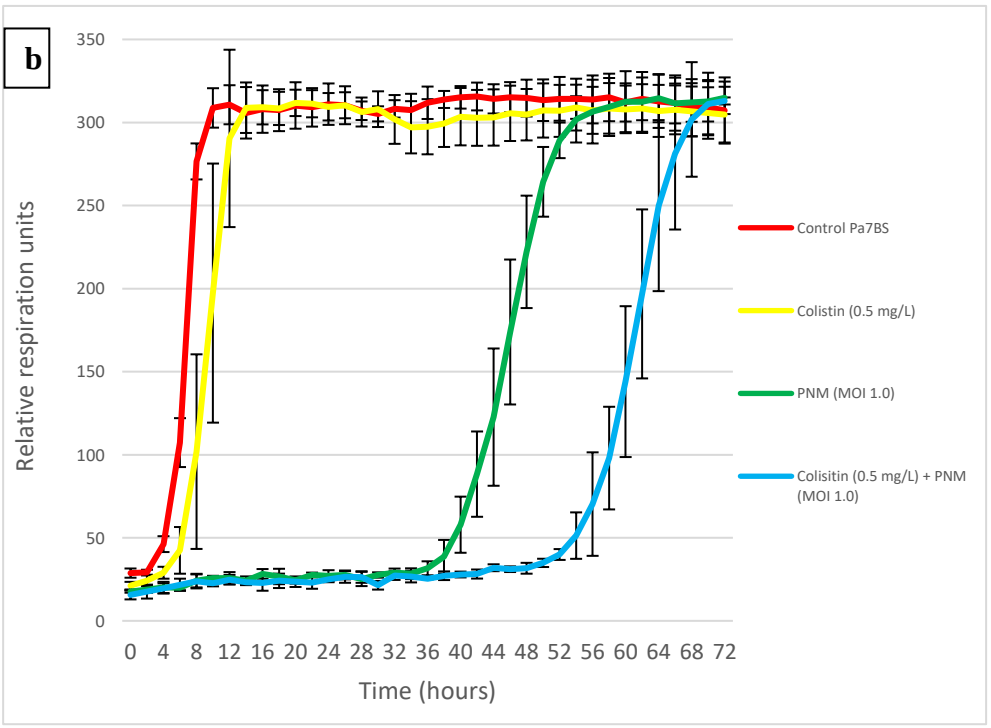
Finally, we should also stress that we were unable to investigate a key component of the co-evolving "predator-prey" duet: the phages.

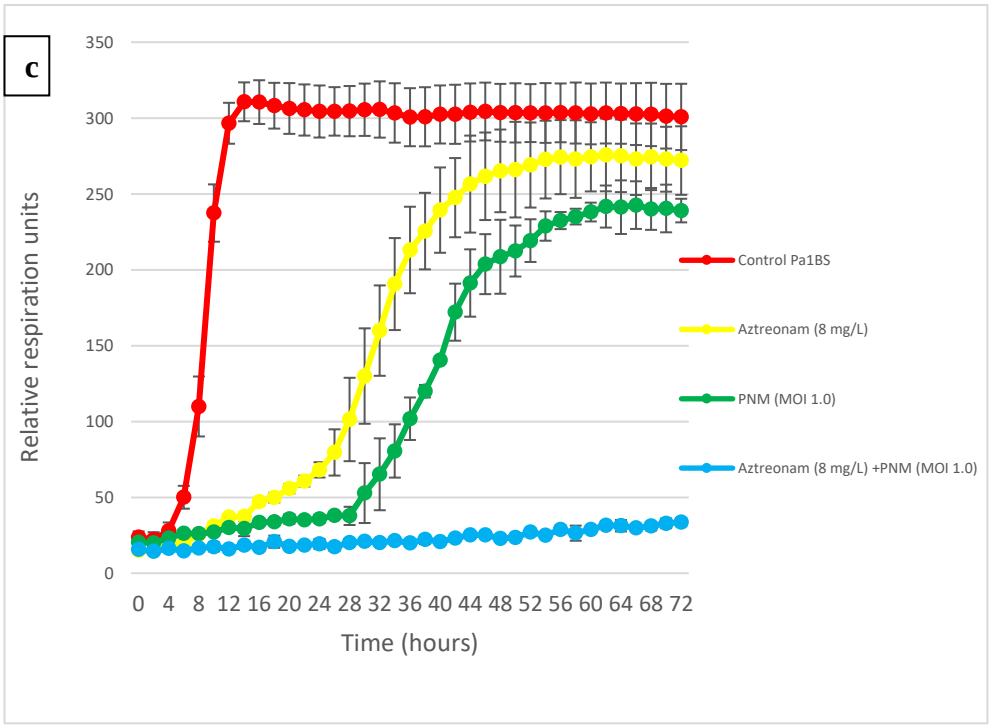
Unfortunately, biological samples that would allow re-isolation of co-evolved phages from the patient's bloodstream or liver abscesses were not kept. As a result, the BPR phenotype is based on the phage clone that was initially used in PT, and which might have evolved *in vivo* to re-infect the emerging phage-resistant *P. aeruginosa* isolates.

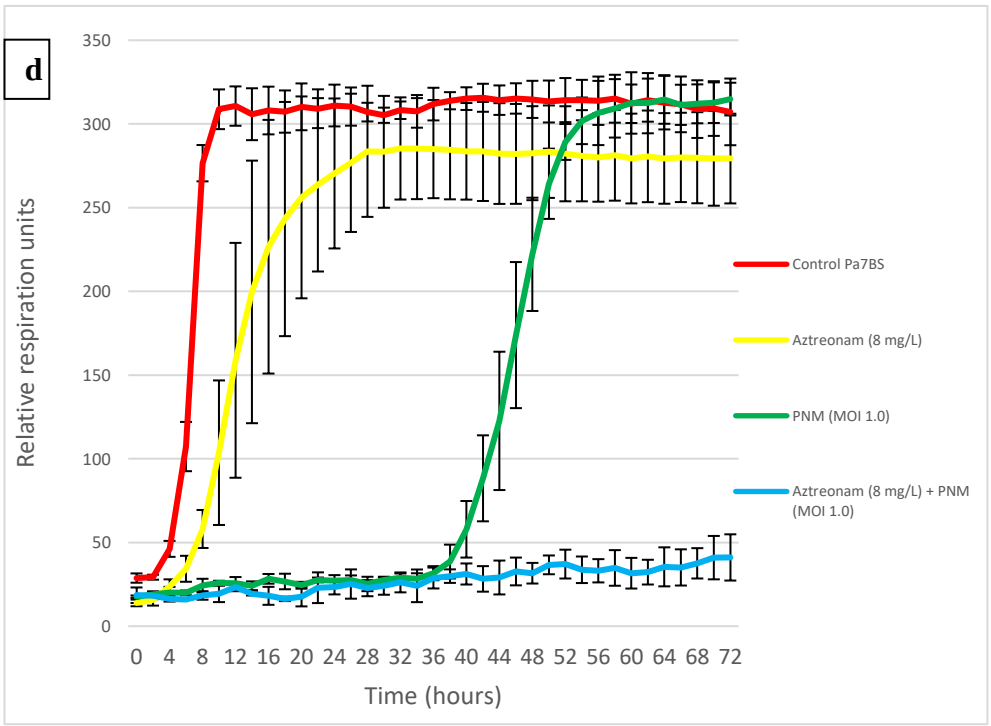
III.1.3.2 Phage-Antibiotic Synergy (PAS)

Even though all PT RCTs that have been performed to date evaluated defined phage products as stand-alone therapies,[146] Phage-Antibiotic Synergy (PAS) is increasingly being reported in the literature,[147] where it is linked to enhanced bacterial killing, increased penetration into biofilms, and decreased selection of antibiotic- or phage-resistant clones. We investigated the possible *in vitro* PAS between phage PNM and sub-optimal concentrations of colistin (0.5 mg/L), aztreonam (8 mg/L), and gentamycin (2 mg/L), which were administered in combination with PT **(Fig. III.1)**. Using an OmniLog® system we showed a clear *in vitro* synergistic activity for phage PNM and any of the three relevant antibiotics against *P. aeruginosa* isolate $Pa1_{BS}$ **(Fig. III.8.a, c, e)**. Similar PAS was observed for isolate $Pa7_{BS}$, although colistin showed slightly less intense synergistic properties **(Fig. III.8.b, d, f)**.

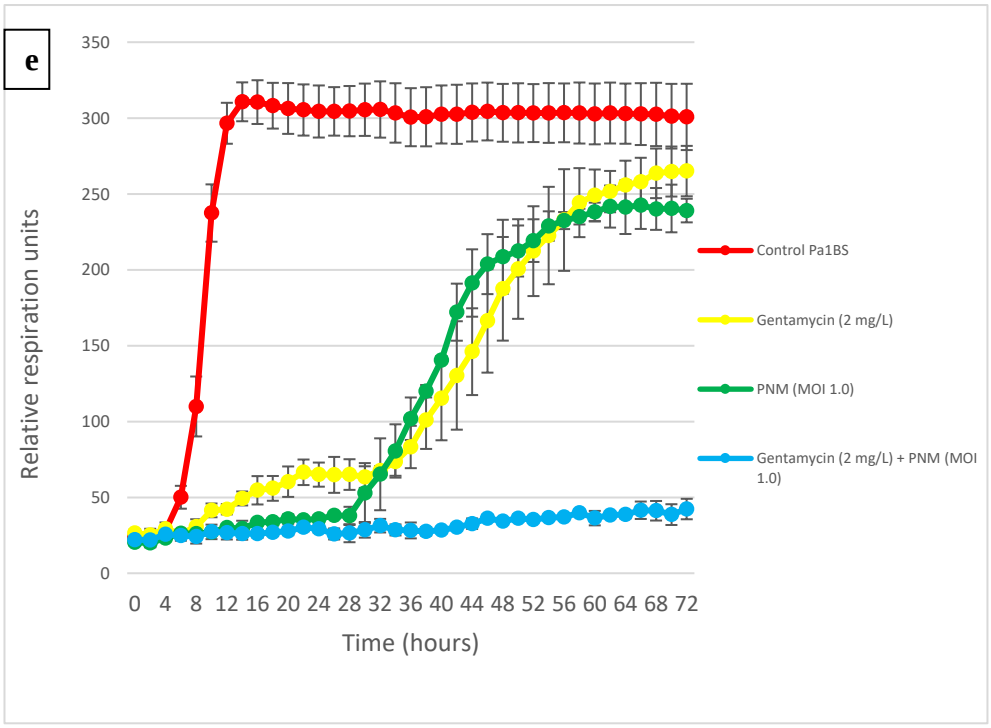

a
Relative respiration units
350
300
250
200
150
100
50
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Time (hours)
Control Pa1BS
Colistin (0.5 mg/L)
PNM (MOI 1.0)
Colistin (0.5 mg/L) +PNM (MOI 1.0)

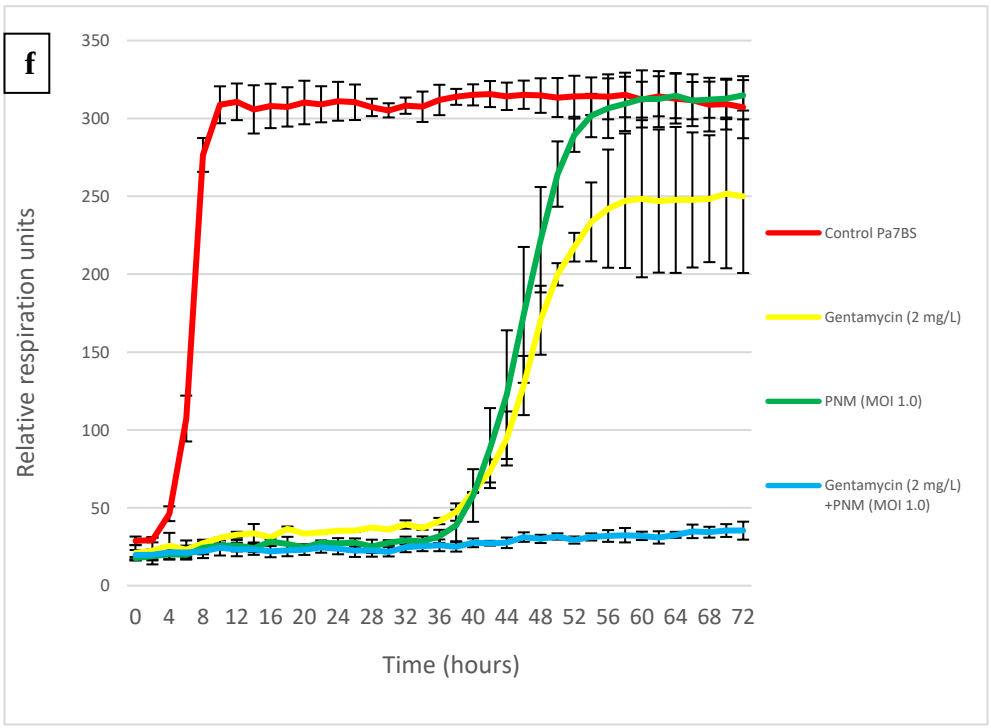

b
Relative respiration units
350
300
250
200
150
100
50
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Time (hours)
Control Pa7BS
Colistin (0.5 mg/L)
PNM (MOI 1.0)
Colisitin (0.5 mg/L) + PNM (MOI 1.0)


c
Relative respiration units
350
300
250
200
150
100
50
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Time (hours)
Control Pa1BS
Aztreonam (8 mg/L)
PNM (MOI 1.0)
Aztreonam (8 mg/L) +PNM (MOI 1.0)


d
Relative respiration units
350
300
250
200
150
100
50
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Time (hours)
Control Pa7BS
Aztreonam (8 mg/L)
PNM (MOI 1.0)
Aztreonam (8 mg/L) + PNM (MOI 1.0)

1960


e
Relative respiration units
350
300
250
200
150
100
50
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Time (hours)
Control Pa1BS
Gentamycin (2 mg/L)
PNM (MOI 1.0)
Gentamycin (2 mg/L) + PNM (MOI 1.0)


f
Relative respiration units
350
300
250
200
150
100
50
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Time (hours)
Control Pa7BS
Gentamycin (2 mg/L)
PNM (MOI 1.0)
Gentamycin (2 mg/L) +PNM (MOI 1.0)

1961

Fig. III.8 | Phage-antibiotic synergy assay results. Activities of phage PNM (at multiplicity of infection [MOI] 1.0) and **a/b** colistin (0.5 mg/L), **c/d** aztreonam (8 mg/L), and **e/f** gentamycin (2 mg/L), as well as combinations of phage PNM and these antibiotics against *Pseudomonas aeruginosa* isolate $Pa1_{BS}$ **(a, c, e)** and $Pa7_{BS}$ **(b, d, f)** were determined using an OmniLog® system. Bacterial proliferation is presented through relative units of cellular respiration. Efficacious phages, antibiotics, and combinations thereof, suppress bacterial proliferation. Results are presented as mean values of three experiments (biological replicates) with error bars representing the standard deviations of the means

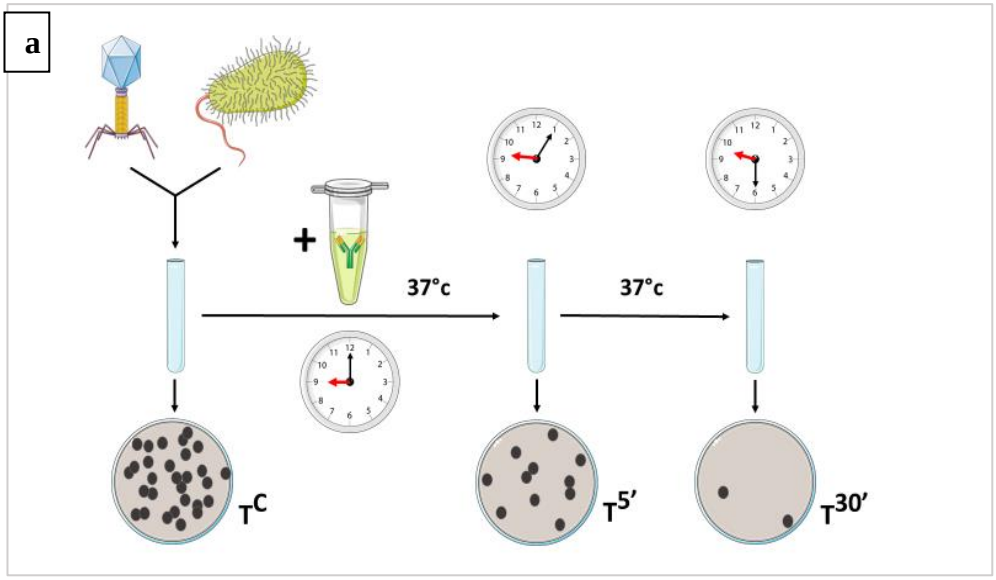
III.1.3.3 Phage Immune Neutralization (PIN)

Phages are able to elicit specific immune reactions in humans that could potentially eliminate the phages and impact PT efficacy[148]. As early as the late 1930's, it was assumed that the appearance of anti-phage antibodies typically could cause the failure of PT, and therefore the application of the same phage(s) for a period longer than two weeks was rarely prescribed in the former Soviet Union[149]. Recent reviews indicate that the presence of phage-specific antibodies can interfere with therapeutic efficacy, though admitting that such phenomenon has not yet been systematically addressed in patients[150]. A study in 20 patients with *S. aureus* infections revealed that PT induces significant anti-phage IgM and, particularly, IgG titers[151]. However, the deteriorating effect of such antibodies on PT's efficacy and on chances of clinical success seems highly dependent on the phages' administration route, their effect being seemingly very limited in cases of topical or digestive

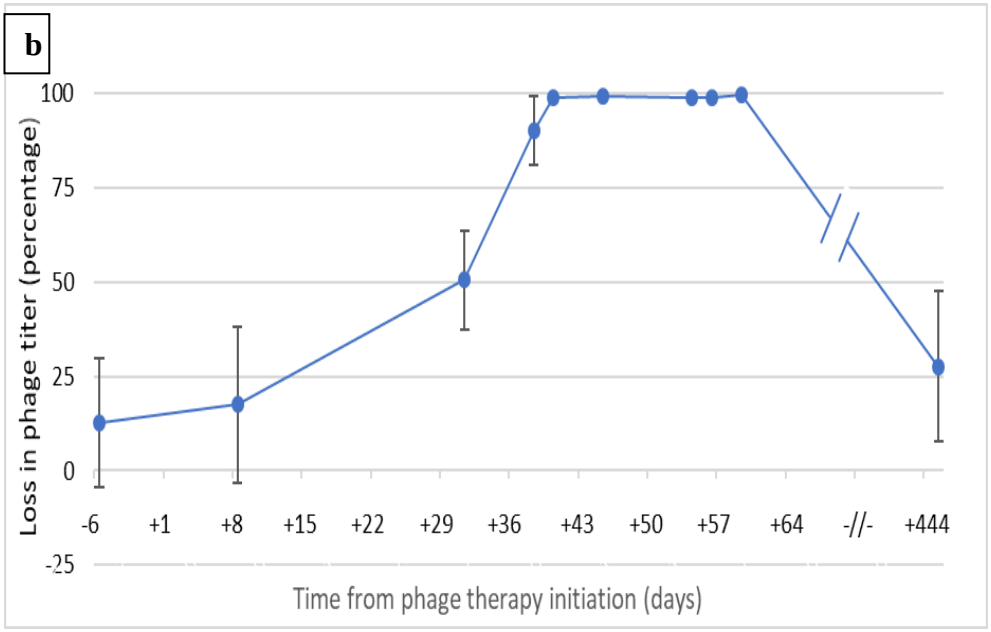
applications[148]. Moreover, the induction of phage-specific antibodies seems irrelevant for PT of acute infections, because antibodies are formed after the phages have performed their antibacterial effect. In contrast, phage-specific antibodies are more relevant in long-term PT of chronic infections or when repeated treatments with the same phages boost the humoral immune response. This was recently confirmed in a case study: an immunocompetent patient with *Mycobacterium abscessus* lung infection was treated for six months with a three-phage cocktail administered intravenously, and the emergence of a potent IgM and IgG neutralizing antibody response to the phages after one month of PT was associated with limited therapeutic efficacy after two months of PT[128].

We assessed the PIN capacity of ten serum samples, one taken prior to PT initiation (control), and the other nine at various time points during and after PT. Phage lytic activity (titer) was determined by double-agar overlay plaque assay, before and after incubation with the patient's serum samples. This assay thus indirectly assesses the PIN effect of serum samples against a given phage strain. The rationale behind this assay is that it is understood that only phage-neutralizing immunoglobulins in a serum sample could be responsible for a time-dependent loss of phage titer. As such, this method does not directly detect specific antibodies against specific phage antigens. Over the entire 51-day period of IV PT covered by the serum samples, no PIN effect was observed against *P. aeruginosa* phages PNM and 14-1. In contrast, *S. aureus* phage ISP, however, was shown to elicit a PIN effect. The chronological evolution of the PIN reaction against ISP is represented in **Figure III.9**. The PIN effect against phage ISP emerged during the fifth week of PT and reached a steady maximum during the sixth week. An additional

2013 serum sample collected approximately one-year after DDLT showed that the
2014 PIN reaction had disappeared by then.



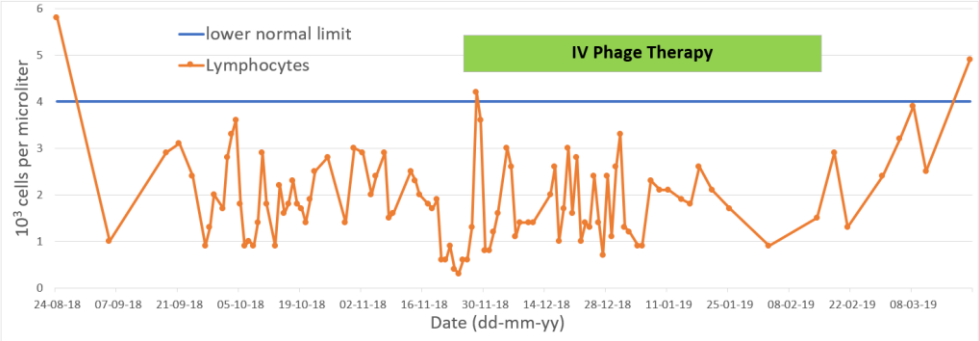
2015



2016

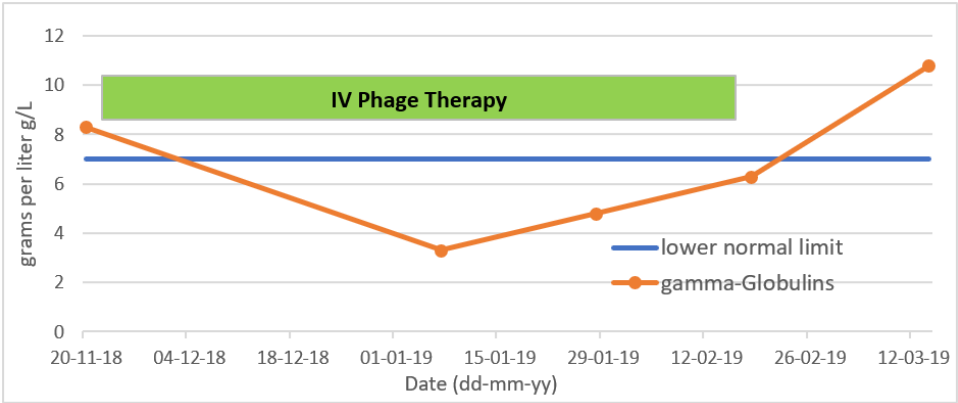
2017

c



2018

d



2019

Fig. III.9 | Phage Immune Neutralization (PIN) assay. a Schematic overview of the assay's methodology (T^{C}: control titer ; $T^{5'}$: titer after 5 minutes of serum-phage co-incubation ; $T^{30'}$: titer after 30 minutes of serum-phage co-incubation). **b** Chronological phage immune neutralization (PIN) activity against phage ISP of ten patient sera collected before, during and after phage therapy. The evolution over time of the PIN activity against phage ISP is shown. […]

[…] Serum PIN activity is shown as % phage titer loss (compared to a pre-PT control serum) after incubation of phage ISP with sequential serum samples for 30 minutes. PIN activity appeared during the fifth week of PT initiation and had disappeared in a one-year post re-transplantation serum sample. Data are presented as mean values with error bars representing the standard deviation of the means. Each serum sample was tested on at least three independent occasions: 6 times for the 24-12-18 sample, 4 times for the 07-01-19 and 18-01-19 samples, and 3 times for all the other samples. **c-d** Persistent biological immunosuppression over the course of PT is illustrated through repeated quantification of the patient's blood lymphocytes (normal values 4.0-10.5x10^3 cells/microliter) and gamma-globulins (normal values 7.0-15.0 grams/liter).

In our experience, the serum PIN pattern expressed by this child is atypical. Indeed, no PIN activity was detected against phages PNM and 14-1 during or after a very long IV application of these phages, whereas unpublished cases did detect potent PIN against PNM and 14-1, already starting significantly during the second week of PT. This could suggest that, at least in this case, this atypical pattern was not related to phage-dependent factors but rather to host-dependent factors, such as the persistent immunosuppression endured by the patient during the entire duration of PT[152]. Indeed, repeated serum gamma-globulin and blood lymphocyte quantifications indicate that the child endured a sustained lymphopenia whose degree of severity varied over time, with the most severe stages being observed during the onset of bacteremic episodes, such as the *P. aeruginosa*

bacteremia starting four days before PT initiation **(Fig. III.9.c & d)**. The child's general immune-incompetence, and his lymphopenia in particular, are most probably multifactorial. A considerable body of work has documented a constitutional immune immaturity in children, especially of the adaptive mechanisms, including an immaturity of T-independent B-cell activation up to the age of five years[153]. In addition, since the child was treated with corticosteroids and tacrolimus since his first liver transplantation, the drug-induced component of the lymphopenia and suspected lymphocytic malfunction should not be underestimated. The most severe stages of lymphopenia (reaching lows of <300 cells/µL) are likely linked to the bacterial sepsis episodes and associated apoptosis-induced lymphopenia[154]. The fact that phage PNM, which showed an *in vitro* lytic activity against the patient's *P. aeruginosa* strain, did not elicit a PIN reaction in this immunosuppressed child was fortunate, as it allowed a prolonged (86 days) therapeutic and prophylactic IV application of BFC1. The observation that phage ISP was able to elicit PIN in this immunocompromised patient does not seem to be due to especially potent immunomodulatory properties of phage ISP compared to those of phage 14-1 or phage PNM[155]. We did not assess whether higher intrinsic immunogenicity of some surface epitopes of phage ISP, like specific glycoproteins, might explain this phenomenon[156].

III.1.3.4 Conclusion

In conclusion, long-term (86 days) IV PT was shown to be safe in this vulnerable young child with uncontrolled *P. aeruginosa* bacteremia, advanced biliary disease, and immunosuppression. PT initiation was

2058 associated with a marked and sustained improvement of the child's clinical
2059 condition, and resulted a few days later in the definitive eradication of *P.*
2060 *aeruginosa* from the blood stream. This favorable progression enabled a
2061 dearly needed liver re-transplantation. Five years later, the child remains well
2062 and has not suffered any new infection. This is the longest reported IV PT in
2063 a child and, to our knowledge, also the first time that PT instillation was
2064 performed during the anhepatic phase of liver transplantation surgery. It is
2065 known that BPR, PAS, and PIN can occur and can have an impact on PT. We
2066 observed BPR against native phage PNM in four out of seven *P. aeruginosa*
2067 isolates retrieved from both the bloodstream and liver abscess pus. Neither
2068 the use of a *Galleria mellonella* virulence model nor the use of a HeLa cell
2069 virulence model allowed us to illustrate phage-induced virulence tradeoffs
2070 directly between the phage-susceptible and phage-resistant bacterial
2071 subpopulations, but the *Galleria mellonella* assay did show a significantly
2072 higher virulence of liver-borne isolates compared to bloodborne isolates.
2073 Whether this difference is linked to a difference in the degree of exposure to
2074 phages in these bacterial subpopulations is not certain, and this clustering was
2075 not observed in the HeLa cell virulence assay. The results of these assays
2076 possibly suffer from survivor bias. Choosing a valuable model on a case-by-
2077 case basis to investigate possible PIVT is an experimental challenge that
2078 warrants further research, and should probably be based on bacterial species,
2079 phage receptor and the contribution of this receptor to the bacterial virulence
2080 phenotype. *In vitro* PAS was observed for phage PNM in combination with
2081 any of the three antipseudomonal antibiotics that were administered
2082 simultaneously: colistin, aztreonam and gentamycin. Finally, PIN reaction
2083 was considered incomplete, as it fortunately did not develop against anti-*P.*

aeruginosa phages PNM and 14-1. This is likely due to patient-dependent factors including a multifactorial severe lymphopenia during the entire duration of PT. In-patient phage pharmacokinetics were however not directly measured, which mitigates the accurate evaluation of PIN and PAS contribution to the patient's outcome, as these mechanisms affect or rely on in-patient phage titers. In the light of the antimicrobial resistance crisis and the paucity of novel antibiotics, PT represents a noteworthy additional tool, in synergy with last-resort antibiotics, to treat challenging infections in patients, including organ transplant recipients and children.

III.1.4 Methods

III.1.4.1 Antibiotic susceptibility

At the QAMH, antibiotic susceptibilities of *the P. aeruginosa* isolates were ascertained using the VITEK 2 system (bioMérieux). At Saint-Luc University Hospital, antibiotic susceptibilities were ascertained through disk diffusion method using Adagio reading technology (Bio-Rad, Marnes-la-Coquette, France) and through automated microdilutions (Phoenix, Becton Dickinson, Franklin Lakes, NJ, USA). Categorization (therapeutic interpretation) of Minimum Inhibitory Concentrations (MICs) were based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

III.1.4.2 Genome sequencing and analysis

The genomes of the seven *P. aeruginosa* isolates were sequenced as previously described[137]. Total genomic DNA (gDNA) was extracted from the isolates with the DNeasy UltraClean Microbial kit (Qiagen) according to the manufacturer's instructions. The gDNA was subsequently prepared for Illumina sequencing using the Nextera Flex (Illumina) and sequenced on an Illumina MiniSeq machine using a paired-end approach (2*150 bp). In addition, the gDNA was also prepared for long read sequencing using the Rapid barcoding kit SQK-RBK004 (Oxford Nanopore Technology) and sequenced on a MinION equipped with a R9.4.1 flowcell (Oxford Nanopore Technology). The quality of the Illumina sequencing data was assessed using FastQC v0.11.9 and trimmomatic[157] v0.39 for adapter clipping, quality trimming (LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15), and minimum length exclusion (>50 bp). Quality of the Nanopore reads was assessed using Nanoplot[158] v1.30, and Porechop v0.2.3 was used for barcode clipping and NanoFilt[158] v2.7.1 for filtering on quality (Q>8) and length (>500 bp). The genomes were reconstructed using the hybrid assembler Unicycler[159] v0.4.8. SNP calling was done using snippy v4.6.0 against the NCBI PGAP[160] annotation of isolate $Pa3_{LS}$. Large structural variations were inspected using ngmlr v0.2.7 and sniffles[161] v1.0.12. The assemblies were visually inspected using Bandage[162] v0.8.1. Serotyping and sequence typing were performed using PAst[163] (accessed in April 2020) and the mlst software v2.19.0. Further functional annotation, antibiotics resistance genes, prophage elements, and genomic islands were annotated using respectively eggNOG-mapper[164] v2, abricate v1, PHASTER[165] (accessed in April 2020), and islandviewer[166] 4. All sequencing data generated in this study has been

deposited in the NCBI BioProject database accession PRJNA776240 [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA776240].

III.1.4.3 *Galleria mellonella* virulence assay

The seven retained *P. aeruginosa* isolates ($Pa1_{BS}$ to $Pa7_{BS}$) were cultured and then diluted in lysogeny broth (LB; 10 g/L tryptone (Neogen, Lansing, Michigan, USA), 5 g/L yeast extract (Neogen), 10 g/L NaCl (Acros Organics, Geel, Belgium)) until reaching an optical density (at wavelength 600 nm) of 0.25 to 0.35. Each bacterial suspension was then centrifugated and the bacterial pellet was re-suspended in phosphate-buffered saline (PBS) (self-made, composition: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, all components from Sigma-Aldrich, Merck KGaA group, 64293 Darmstad, Germany). The PBS suspension was diluted (5.10^{-6} dilution factor) in PBS and 20 µL, representing an inoculum of approximately ten colony forming units (CFU), was injected in a *G. mellonella* larva. The larvae were all of the same age and were separated in eight batches of larvae (one batch for each of the seven *P. aeruginosa* isolates, one for the PBS control. The assay was first calibrated using batches of five larvae, to standardize methodology of injection and estimate intra-batch variance as well as optimal timeframe for follow-up. The assay was then performed with batches of ten larvae and each batch was standardized for weight. Each batch of ten *G. mellonella* larvae was inoculated with one of the seven *P. aeruginosa* isolates, and one batch was inoculated with 20 µL of PBS (control). Intrahemocoelic injection was performed at the basis of the hindmost left pro-leg of each larva, using thin intradermal needles (Micro-fine + insulin U100 0.3 ml syringe,

Becton Dickinson, Franklin Lakes, New Jersey, USA). The *G. mellonella* larvae were stored in petri dishes (one dish per batch) and kept in the dark at 37 °C. Each larva was inspected visually every hour for 16 hours to assess its activity score. The activity score ranges from 0 to 9 points **(Fig. III.7)**.

III.1.4.4 HeLa cell viability assay

The virulence of the *P. aeruginosa* isolates against HeLa cells was assessed via a cell viability assay, based on the protocol described in Hernandez – Padilla et al. (2017)[167]. Briefly, human cells were cultured in Dulbecco's modified eagle medium[1] (DMEM) supplemented with 10 % fetal bovine serum[1] (FBS) and 1 % antibiotic - antimycotic solution[2] (Referred to as DMEM+10, [1]Thermo Fisher Scientific, [2]Sigma Aldrich) and incubated at 37 °C with 5 % CO_2. When confluency was reached, 3 x 10^4 cells were seeded in a 96-well plate and incubated overnight. Next, 10^6 bacterial cells in DMEM+10 lacking phenol red were collected based on optical density ($OD_{600\ nm}$ of 0.5 corresponds to 2 x 10^8 cells) and added to the HeLa cells in a final volume of 200 µL. The following day, the bacterial cells were removed and the medium was refreshed. According to the protocol of the manufacturer, 10 µL MTT (Sigma Aldrich) was added to the cells and incubated for four hours. Finally, 100 µL solubilization buffer (Sigma Aldrich) was added to dissolve the crystals. The viability of the cells was assessed by their ability to cleave MTT (tetrazolium dye) via mitochondrial dehydrogenases. By cleavage of MTT, formazan is formed and is measured spectrophotometrically at 570 nm in a microplate reader (CLARIOstar Plus, BMG Labtech). The experiment was performed with six technological replicates. A Student's t test with the

non-infected HeLa cells as control group was executed to pairwise compare the different treatment groups (significance level $p < 0.05$) using the JMP v16 software (SAS Statistics, Cary, North Carolina, USA).

III.1.4.5 Bacterial growth kinetics

Bacterial cellular respiration was measured using the OmniLog system (Biolog, Hayward, CA, USA). Data was analyzed with OmniLog data Analysis Software (v1.7). Growth kinetics of all seven isolates were first measured in the absence of any phage or antibiotic. Then, to investigate phage-antibiotic synergy, the growth kinetics of *P. aeruginosa* isolates $Pa1_{BS}$ and $Pa7_{BS}$ were assessed in the presence of phage PNM (at MOI 1.0), colistin (0.5 mg/L), aztreonam (8 mg/L), and gentamycin (2 mg/L), and combinations of phage PNM and these antibiotics. Experiments were done in 96-well plates in a final volume of 200 µl of LB supplemented with 100 times diluted tetrazolium dye mix A, according to the manufacturer's instructions. Bacterial cells were added at a concentration of 10^5 CFU/well, calculated based on optical density (OD, at 600 nm) measurements (with an OD of 0.5 corresponding to 4×10^8 CFU/ml, on average), which were validated using a classical plate culture method. The titer of phage PNM was also confirmed after each experiment using the classical double agar overlay method[168]. Plates were incubated at 37 ºC for 72 hours and a possible reduction (causing a color change) of the tetrazolium dye due to bacterial respiration (during growth) was monitored and recorded every 15 min by the Omnilog system. Efficacious phages, antibiotics, or combinations thereof, suppress bacterial

growth (respiration). Experiments were performed in triplicate (biological replicates).

III.1.4.6 Phage immune neutralization (PIN) assay

The PIN activity of the patient's serum samples was determined according to Adams[168] with some modifications. Phage lytic activity (titer) was determined by double-agar overlay plaque assay before and after incubation of the phages with the patient's serum samples. We used a selection of ten serum samples that were harvested from the toddler over a two-month period, from one week before PT initiation to the day 51 of continuous IV PT. Because this assay assesses the PIN capacity of a serum sample against one phage strain, the test was performed for each of the three phage components of BFC1. PIN intensity is correlated to the time-dependent loss of phage titer after incubation with sera. The phage solution is first titrated using its reference host bacterial strain using a standard double-agar overlay titration method, and this titer is used as a control measurement (Tc). Blood was allowed to clot for a minimum of 30 min in a vertical position and then centrifuged at room temperature in a swinging bucket rotor for 10 min at 2,000 x *g*. Serum samples were stored at -80 °C ± 5 °C. To assess the effect of the sequential serum samples on phage lytic activity, 0.9 ml of the diluted sera (1:100 in self-made normal saline, NaCl 0.9%) were mixed with 0.1 ml of the phage at a concentration of 2 x 10^7 pfu/ml and incubated at 37 °C. After 5 min and after 30 min of incubation, the phage titer of the phage-serum mixture is determined using the same double-agar overlay titration method, generating titers T5' and T30', respectively **(Fig. III.9)**. In this method, a

significant loss of titer both between TC and T5' and between T5' and T30’ is highly indicative for the presence of phage-neutralizing antibodies in the tested serum. Conversely, absence of significant differences between titers TC, T5', and T30' suggests an absence of phage-neutralizing antibodies. For each serum sample, the PIN assay was performed between three and six times to generate robust mean results.

III.1.4.7 Statistics & Reproducibility

No data were excluded from the analyses. Each assay is based on at least three independent measurements (n = 3-10 depending to the assay, as described in the main text and in the respective Figure legends). No statistical method was used to predetermine sample sizes. Investigators were blinded to allocation and outcome assessment during the *Galleria mellonella* and HeLa cells assays. The *Galleria mellonella* larvae were standardized for weight and randomly allocated to any of the 8 experimental groups. Randomization was not considered applicable to the other experiments.

III.1.4.8 Additional statements

All sequencing data generated in this study has been deposited in the NCBI BioProject database accession PRJNA776240 [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA776240]. Source data are provided with this paper.

Phages PNM and ISP were originally obtained from Eliava Institute, Tbilisi, Georgia. Phage 14-1 was received from Russia via Professor Krylov, http://eng.genetika.ru/, RIGSIM, current abbreviation.

III.2 Other phage therapy clinical cases

As part of the emerging PT activity of the CUSL, we administered and supervised the PT of six other patients between 2017 and 2023. A summary of the main characteristics of these cases is depicted in **Table III.2** (and ulterior **Table III.3**). The aggregated analysis of these cases is complex as they vary across a series of criteria, such as :

- **Targeted pathogens** (one case combining three of them): *S. aureus* (3), *P. aeruginosa* (3), *E. faecium* (1), *Stenotrophomonas maltophilia* (1)

- General type of **clinical indication** (one case combining all of them): skin and soft tissue infection (3), bone and joint infection (3), intra-abdominal infection (2), respiratory infection (1), bacteremia (1)

- **Route(s) of phage administration** (one case combining all of them): *in loco* instillations (5), topical application (3), intravenous infusion (2), pulmonary nebulization (1)

And by the concomitant administration, in all of these cases, of antibiotics likely affecting the bacterial species targeted by PT. However, such antibiotic therapy had been previously attempted as SOC management in all of these cases prior to PT, and had failed to contain the infection, hence the use of PT. In a similar way to the case described in section III.1 (case 2), some of these cases were more extensively described and graphically summarized (case 1 and case 3) for their previous or upcoming publication, respectively **(Fig. III.10 and III.12)**. We will discuss them in the next sections.

Case #	Age (years)	Sex	Clinical indication	PT-targeted pathogen(s)	AMR profile	Used phage(s)	Phage susceptibility	PT route(s)	AB attempted prior to PT	AB during PT
1	13	F	Chronic pelvic osteomyelitis	***S. aureus***	iMLSB MSSA	ISP	Spot test +	Intra-operative lavage, *in loco* instillations	Clindamycin, rifampin, flucloxacillin	Clindamycin, rifampin, ciprofloxacin
2	1	M	Bacteremia, intra-abdominal infection	***P. aeruginosa***	XDR VIM-2	PNM, 14-1, ISP	PNM EOP = 0.3 Others : 0	Intra-operative lavage, intravenous, biliary instillations	Colistin, gentamycin, aztreonam	Same as prior to PT
3	12	M	Bacteremia, necrotizing fasciitis, arthirtis, pneumonia, intra-abdominal	***S. aureus*** ***P. aeruginosa*** ***S. maltophilia***	MRSA MDR WT	ISP PNM, 14-1, PT07 BUCT700	ISP EOP = 1 Others : spot test +	Intra-operative lavages, intravenous, intra-abdominal & intra-thoracic instillations, pulmonary	For MRSA-mediated necrotizing fasciits and associated bacteremia : vancomycin, clindamycin	Vancomycin, clindamycin, ceftaroline, meropenem, ciprofloxacin, colistin
4	43	F	Intra-abdominal infection	***E. faecium***	VRE vanA	KN	Spot test +	Intravenous, intra-abdominal instillations	Multiple courses of Linezolid	Linezolid, Vancomycin
5	1.5	F	Necrotizing cellulitis	***P. aeruginosa***	WT	C, 4P	Spot test +	Intra-operative lavage, topical	Piperacillin-tazobactam, amikacin	Same as prior to PT
6	17	M	Sub-cutaneous surgical scar infection	***S. aureus***	WT (MSSA)	ISP	Spot test +	Intra-operative lavage, *in loco* instillations	Multiple attempts : oral flucloxacillin, doxycyclin, clindamycin, ciprofloxacin, cotrimoxazole	Clindamycin, cefazolin
7	70	M	Chronic femoral osteomyelitis	***P. aeruginosa***	WT	DP1, 4P	EOP = 0.01 each	Intra-medullary instillations	Multiple attempts : meropenem, ceftazidime, piperacillin-tazobactam, ciprofloxacin	Ceftazidim

Table III.2 | Main descriptive characteristics of the 7 phage therapy cases. Additional features of these cases are depicted in ulterior Table III.3. PT, phage therapy; AB, antibiotic; WT, wild type; AMR, antimicrobial resistance; iMLSB, inducible macrolides-lincosamides-streptogramin B; MRSA/MSSA, methicillin-resistant/susceptible *S. aureus*; MDR, multidrug resistant; XDR, extensively drug resistant; VIM-2, Verona integron-encoded metallo-beta-lactamase type 2; VRE, vancomycin-resistant *Enterococcus*.

III.2.1 Case 1 – *S. aureus* chronic osteomyelitis[130]

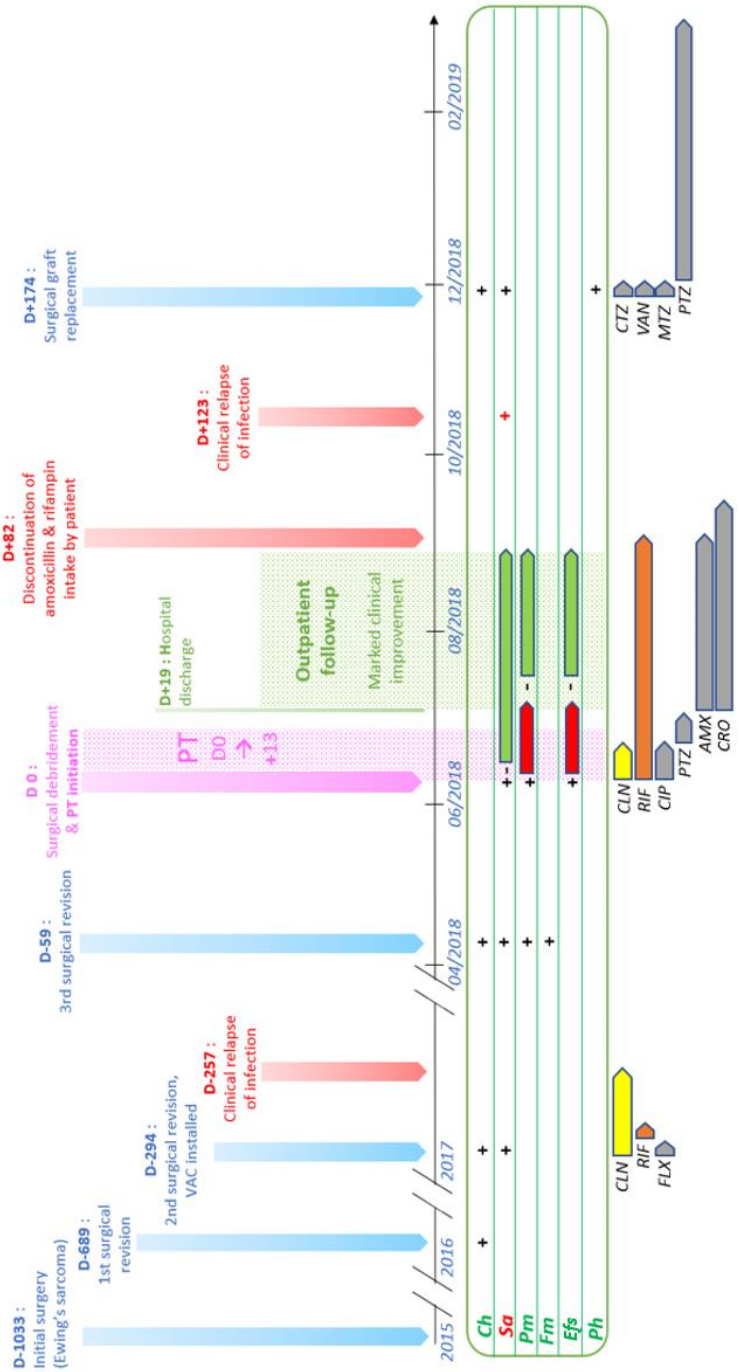


Fig. III.10 | Case 1 - Timeline of the most relevant events, procedures and treatments in the patient's history, from initial Ewing sarcoma surgical resection to termination of antibiotic therapy after final surgical graft replacement. From top to bottom: vertical arrows represent important events in the clinical progression of the case. Blue vertical arrows represent surgical procedures. *VAC, vacuum-assisted closure ; PT, phage therapy.* Microbiological culture results are displayed in the green horizontal box. Horizontal arrows in this box represent continuation of similar result over repeated daily analysis. *Ch, Clostridium hathewayi ; Sa, Staphylococcus aureus ; Pm, Proteus mirabilis ; Fm, Finegoldia magna ; Ef, Enterococcus faecalis ; Ph, Peptoniphilus harei.* Lower horizontal arrows represent antibiotic therapies. *CLN,* clindamycin ; *RIF,* rifampin *; FLX,* flucloxacillin ; *CIP,* ciprofloxacin ; *PTZ,* piperacillin-tazobactam ; *AMX,* amoxicillin ; *CTX,* ceftriaxone ; *CTZ,* ceftazidime ; *VAN,* vancomycin ; *MTZ,* metronidazole.

III.2.1.1 General presentation and discussion of Case 1

We here report the case of a 13-year-old girl who developed chronic polymicrobial biofilm infection of a pelvic bone allograft after Ewing's sarcoma resection surgery. Chronic infection by *Clostridium hathewayi, Proteus mirabilis* and *Finegoldia magna* was worsened by MRSA exhibiting inducible Macrolides-Lincosamides-Streptogramin B resistance phenotype (iMLSB). After failure of conventional conservative treatment, we initiated *in situ* anti-*S. aureus* PT using phage ISP at titer 10^7 PFU/mL, by performing

intra-operative phage lavage (50mL during 15 minutes) and subsequent instillations by "pig tail" drainage catheters (40mL three times daily for one week, 30 mL two times daily for one additional week). All of these phage applications were preceded by sodium bicarbonate solution pre-treatment. Re-aspirates of this sodium bicarbonate solution from the drainage catheter (prior to each phage application) were sent once daily to microbiological analysis, documenting the disappearance or persistence of the locally-infecting pathogens **(Fig. III.10)**. This was combined with surgical debridement and intravenous antibiotic therapy. This led to marked clinical and microbiological improvement, yet failed to prevent a recurrence of infection on the midterm, which eventually led to surgical graft replacement.

This complex case of a bone allograft polymicrobial osteomyelitis highlights the difficulties of treating this type of infection with conventional therapy, but also with PT. Multiple causes could explain the therapeutic failure of conventional treatment. First, the chronicity of the *Clostridium hathewayi* infection, with an infection that has been detected for more than two years at the time of PT initiation; then the acutization of the infection with *S. aureus*, a bacterial species known to create biofilms and therefore difficult to treat with conventional antibiotics; the polymicrobial nature of the osteitis requiring a combination of several antibiotics, leading to poor clinical tolerance; and lastly, the acquisition of antibiotic resistance mechanisms. In addition, local postoperative radiation therapy could have hindered antibiotic pharmacokinetics by limiting their distribution to the infected site. Indeed, radiation-induced fibrosis has been reported to deteriorate microvascular circulation in both bone and skin and soft tissues, which could result in poorer *in situ* distribution and effect of intravenous antibiotics[169, 170].

While all *S. aureus* isolates cultured were showing a MSSA phenotype, they were also exhibiting an iMLSB phenotype, which was assessed by D-test[171]. Detection of this iMLSB phenotype was considered a relative contraindication to the reintroduction of clindamycin, an antibiotic of the lincosamides family, to the antibiotic treatment of the patient. Indeed, its administration could induce rapid resistance to MLSB antibiotics in *S. aureus*, which could result in therapeutic failure and loss of valuable therapeutic options. These reasons made us ultimately consider PT as last resort conservative treatment.

Introduction of PT in addition to antibiotics and surgical debridement yielded contrasting results in this patient. On the one hand, the situation made promising progress on the short term after PT initiation, on both the microbiological and the clinical plan. On the microbiological plan, culture of deep liquid aspirations samples from the infected wound remained negative for *S. aureus*, which the PT specifically targeted, during the whole course of PT and up to at least two months after it was discontinued. On the clinical plan, PT initiation correlated with a rapid decrease in local and biological inflammatory features with favorable evolution of CRP levels from 125 mg/L after 48 hours of PT to 8 mg/L after 14 days of PT (normal values <5 mg/L).

However, mid-term progression was less favorable. Three months after PT discontinuation, recurrence of the infection was diagnosed. Pus culture were positive for a similar phenotype of *S. aureus*. This could suggest a relapse of the infection caused by the same strain as the one we isolated before PT initiation, yet does not totally rule out the possibility of a

reinfection with a different *S. aureus* strain that would harbor the same MSSA/iMLSB phenotype.

Several factors are to be considered to explain the post-PT relapse of infection in our patient. Compliance with post-operative antibiotic therapy was poor, antibiotic intake being unilaterally discontinued by the patient after two months instead of the recommended three months. Moreover, the postulate under which PT was initiated was likely flawed. Although the infection in our patient was polymicrobial, LabMCT could provide us phages targeting only for *S. aureus.* Despite extensive research, including in foreign laboratories, we were unable to obtain phages active against *Clostridium hathewayi*, *Finegoldia magna* or *Proteus mirabilis*. Nevertheless, we initiated PT against *S. aureus* only, believing that eradicating the more acute and aggressive pathogen could have an impact on the broader organization of the chronic polymicrobial biofilm. This "kill-the-leader" strategy did not pay off. Since several bacterial species were not covered by PT, we hypothesize that the majority of the polymicrobial infection and its biofilm configuration were unaffected by this combined treatment, and eventually remained a suitable terrain for new *S. aureus* superinfection. This eventually brought the situation back to its initial dead-end after four months of respite. Recent attempts at treating orthopedic infections with PT suggest that PT should be used in combination with antibiotics and proper surgical care ; they also seem to achieve durable clinical and microbiological successes by focusing on mono- or dual-species biofilms where full phage coverage of the pathogens can be achieved[172, 173, 174, 175]. In our case, full coverage of the polymicrobial infection by PT was not possible.

Several limitations to our analysis of the case are to be noted. First, we did not keep any *S. aureus* strain retrieved after PT, either when it was first detected on pus swab culture upon infection relapse, or when it was detected on the intraoperative samples collected during surgical graft replacement. Comparing phage susceptibility between those strains and the initial *S. aureus* strain on which our investigations were based could have highlighted mechanisms of phage-resistance acquisition. We also did not keep any serum sample that would have allowed us to search for the development of phage-directed humoral immune response. However, limiting PT duration to two weeks likely mitigates the risk of immune neutralization development, even though *in situ* local PT is notably able to induce it past this term[148]. Nevertheless, these reactions should not automatically be considered a hurdle for *in situ* PT's efficacy[148].

III.2.1.2 Phage-Antibiotic Interactions in Case 1

Assessment of potential interactions between phages and concomitant antibiotics through OmniLog® assays provided interesting results **(Fig. III.11)**: we will further discuss the case of phage-clindamycin and phage-rifampin interactions.

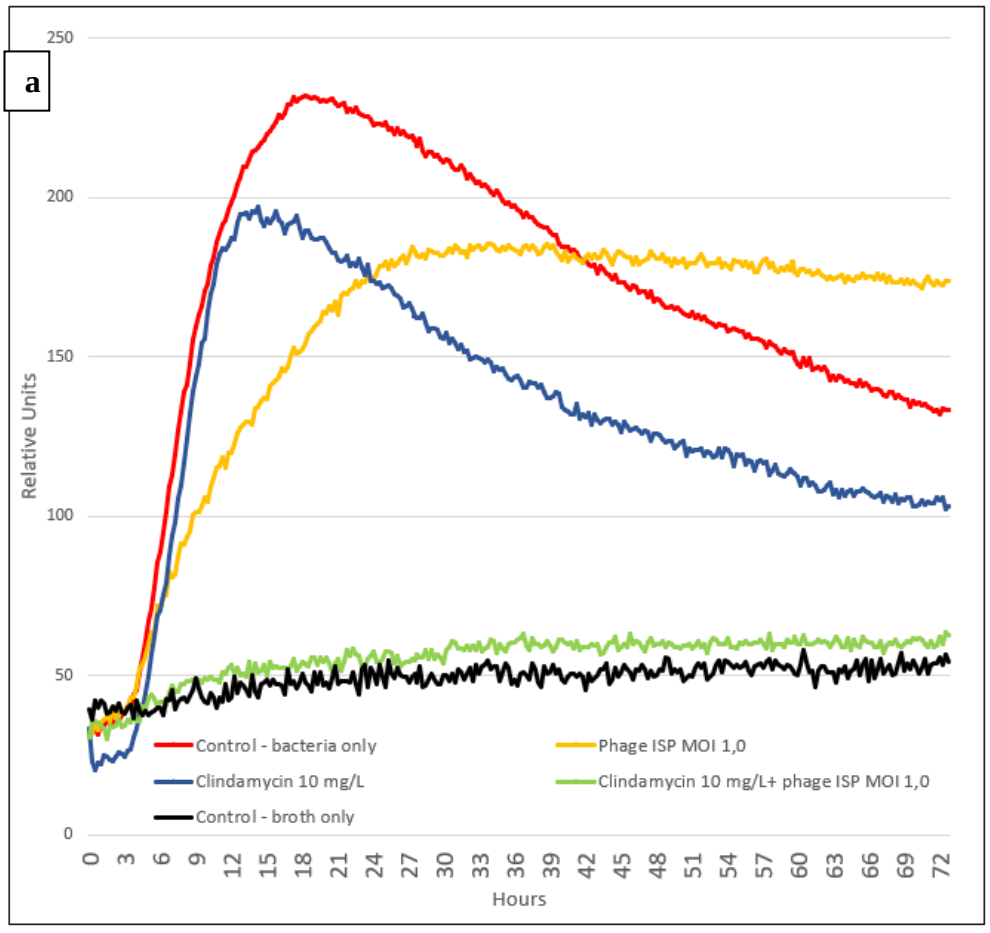
Adjunction of phage ISP at titer MOI 1 had seemingly synergistic properties with clindamycin, possibly conferring re-sensitization to high clindamycin concentrations of 10 mg/L **(Fig. III.11.a)**. Potential explanation to this phenomenon might be found at the molecular and metabolic level in *S. aureus*. As explained, this *S. aureus* strain exhibits resistance to clindamycin

through an iMLSB phenotype. This phenotype is caused by the expression of mostly plasmid-borne *erm*-family genes, encoding for Erm-family methyltransferases that confer protective methylation to the molecular target of macrolides, lincosamides and streptogramin B: the 23S ribosomal RNA (rRNA) of the larger 50S ribosomal subunit. In iMLSB phenotype, inducibility mechanisms towards the *erm*-family gene reside at the post-transcriptional level. Schematically, the *erm*-family gene is transcribed in a cognate mRNA molecule whose ribosomal translation is then blocked by a "leader peptide", preventing the production of methyltransferases ; "inducer" molecules, such as erythromycin, can in turn block the leader peptide's translation, which allows unbridled translation of the *erm*-mRNA into methyltransferases, leading to the development of iMLSB resistance phenotype[176]. Though it has been subject to contradicting claims, recent experiments show that erythromycin is not the only antibiotic able to induce Erm methyltransferase production through leader peptide repression: clindamycin itself, *inter alia*, can exert similar activity on the ErmB methyltransferase-ErmBL leader peptide couple, though not in the exact same way as erythromycin[176, 177, 178]. Consistent with this hypothesis, no concentration of clindamycin alone could suppress peak bacterial growth in OmniLog® culture, suggesting clindamycin-resistance had been induced by clindamycin itself. In the patient's *S. aureus* strain, clindamycin possibly induces unbridled translation of *ermB*-mRNA into ErmB methyltransferases, rapidly leading to the acquisition of a clindamycin-resistant phenotype. Yet, adjunction of phage ISP at MOI 1 seems to reduce the resistance-inducing power of clindamycin, this combination leading to a marked and durable suppression of bacterial growth. This could be explained by the fact that

phage ISP, as a virus, diverts ribosomal activity for its own needs of progeny virion production and completion of lytic cycle[179]. This subversion of the ribosomal function can have dramatic effects on bacterial proteins translation, at times suppressing it totally[180]. In our case, phage ISP-mediated subversion of bacterial ribosomal function possibly prevents translation of *ermB*-mRNA, even though clindamycin has released it from ErmBL sequestration. This would result in reduced or suppressed ErmB methyltransferase activity, which could explain how phage ISP reduces the resistance-inducing properties of clindamycin in certain iMLSB *S. aureus*.

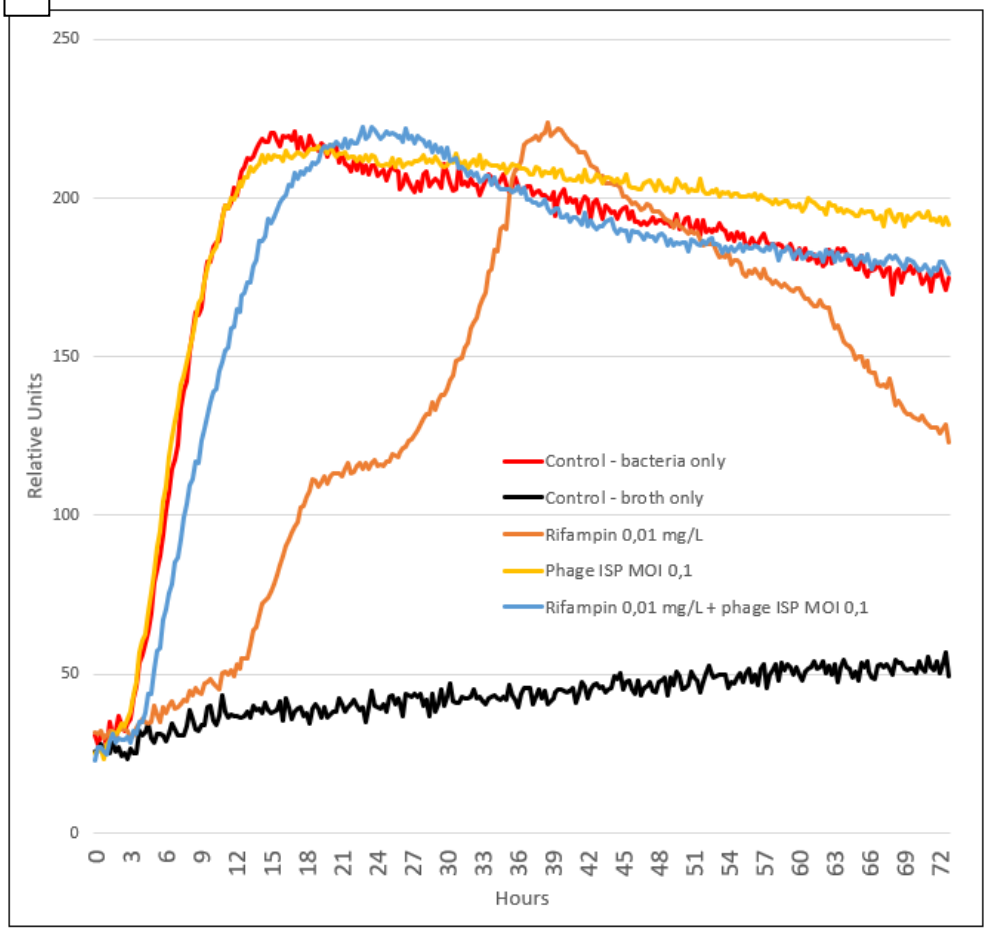
Conversely, combination of phage ISP with rifampin did not result in synergistic effect **(Fig. III.11.b)**. Furthermore, breakpoint concentrations of rifampin combined with sub-inhibitory phage titer of MOI 0.1 did appear to provide a moderately weaker growth-inhibitory effect than the same breakpoint concentration of rifampin used alone. Molecular basis for antagonistic pharmacodynamics between phages and rifampin do exist. Rifampin's bactericidal properties are based on its inhibitory properties towards bacterial RNA polymerase[181]. However, phage ISP does not encode for its own RNA polymerase, and fully depends on its bacterial host's RNA polymerase for transcription[182]. Rifampin's effect on ISP's bacterial host could thus prevent phage genome transcription, resulting in antagonistic effects. Consistent with this hypothesis, rifampin's inhibitory effect on some phages' RNA synthesis had already been documented decades ago[183]. More recently, review of literature has presented numerous occurrences of pre-clinical studies documenting rifampin's inhibitory effects on phage infectivity[184]. Yet, evidence suggests that the possibility of phage-rifampin synergy should not be ruled out in specific conditions, for example through

combined use of rifampin and phage Sb-1 in rifampin-susceptible *S. aureus* biofilms[185]. However, as opposed to phage ISP, sequencing data suggests phage Sb-1's genome encodes for an RNA polymerase[186].



2510

b



2511

2512

2513

2514

2515

2516

c

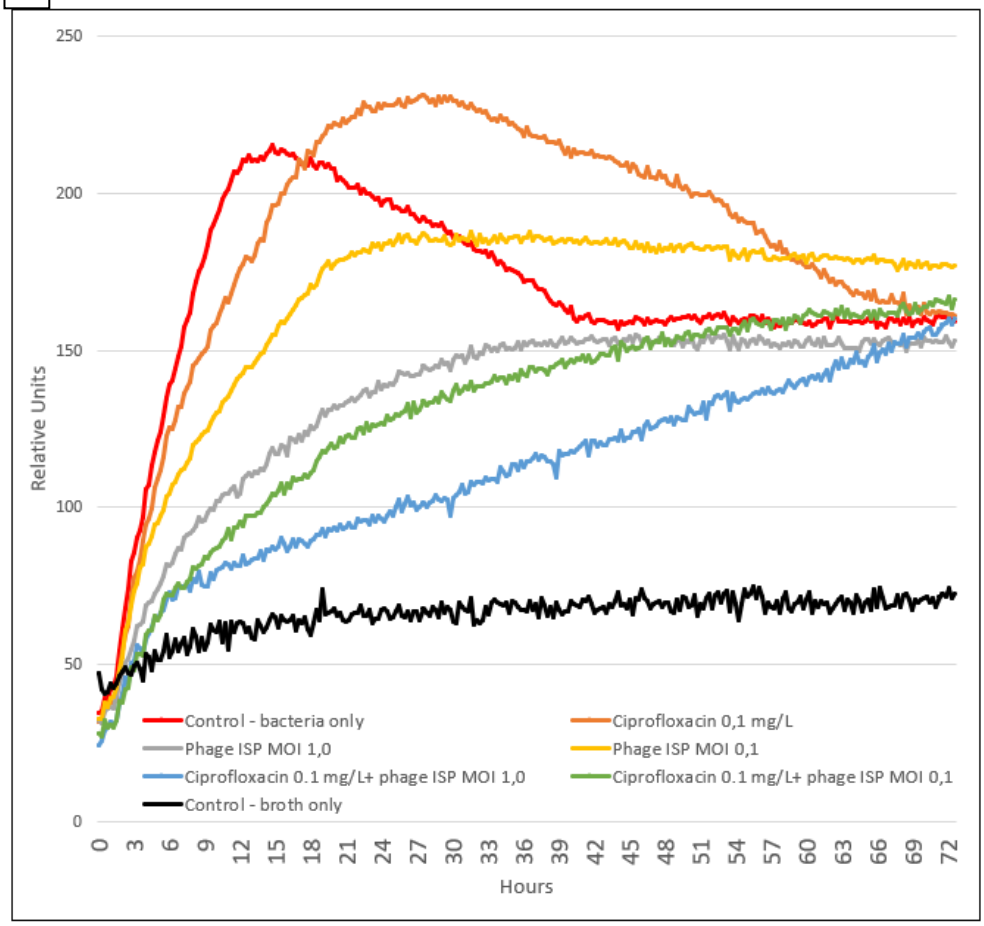


Fig. III.11 | Case 1 - Assessment of phage-antibiotic interactive properties by OmniLog® system. Kinetics of bacterial growth under various conditions is expressed as Relative Units over a 72 hours timeframe. Positive and negative controls were systematically measured using respectively bacteria only with no antibacterial agent, and tryptic soy broth only without bacteria. Phage ISP at various Multiplicity Of Infection (MOI) values was combined

with different concentrations of the three antibiotics used concomittantly in the patient: **(a)** clindamycin ; **(b)** rifampin ; **(c)** ciprofloxacin.

Lastly, we should acknowledge that although these two phage-antibiotic interactions have likely taken place *in vitro*, whether they played any role *in vivo* regarding the case's progression remains uncertain. Several limitations of interpretation shall be noted. First, OmniLog® assays analyze single species bacterial growth in short-term liquid phase culture. To what extent the results obtained from these assays can be transposed to the pharmacodynamics and pharmacokinetics that took place in a chronic, biofilm-organized polymicrobial infection is unpredictable. Similarly, interactive properties in OmniLog® assays depend on both specific antibiotic concentrations and phage titers, the combination of which might not have been achieved *in vivo*. As all reagents were added to the microplates simultaneously, our OmniLog® assays also did not investigate the effect of chronological order between phage's and antibiotics' introduction to the bacterial culture. This limitation warrants further investigation, as recent *in vitro* evidence advocating for phage-antibiotic synergies in *S. aureus* biofilm strongly suggests that different orders of introduction between phages and antibiotics can result in significantly different bactericidal effects, favoring a "phage first" approach for staggered phage-antibiotic administrations[187]. Recent clinical application of these findings came to the same conclusion[175].

III.2.1.3 Conclusion – Case 1

Combination of *in situ* anti-*S. aureus* PT, intravenous antibiotics and surgical debridement was used as a salvage therapy for a polymicrobial chronic osteitis of a pelvic bone allograft. This led to favorable results on the short term on both the microbiological and clinical plan, yet failed to prevent a recurrence of infection on the midterm. This eventually led to surgical graft replacement. The reasons explaining this midterm failure are multifactorial, including poor compliance with antibiotics and incomplete coverage of the polymicrobial infection by PT. Phage-antibiotics interactive properties should not be considered "unconditionally synergistic", and should be assessed in a case-by-case manner depending on antibiotic type, antibiotic concentration and phage titer. Specific pharmacodynamics of phages and antibiotics might explain these differences.

III.2.2 Case 3 – *S. aureus* necrotizing fasciitis

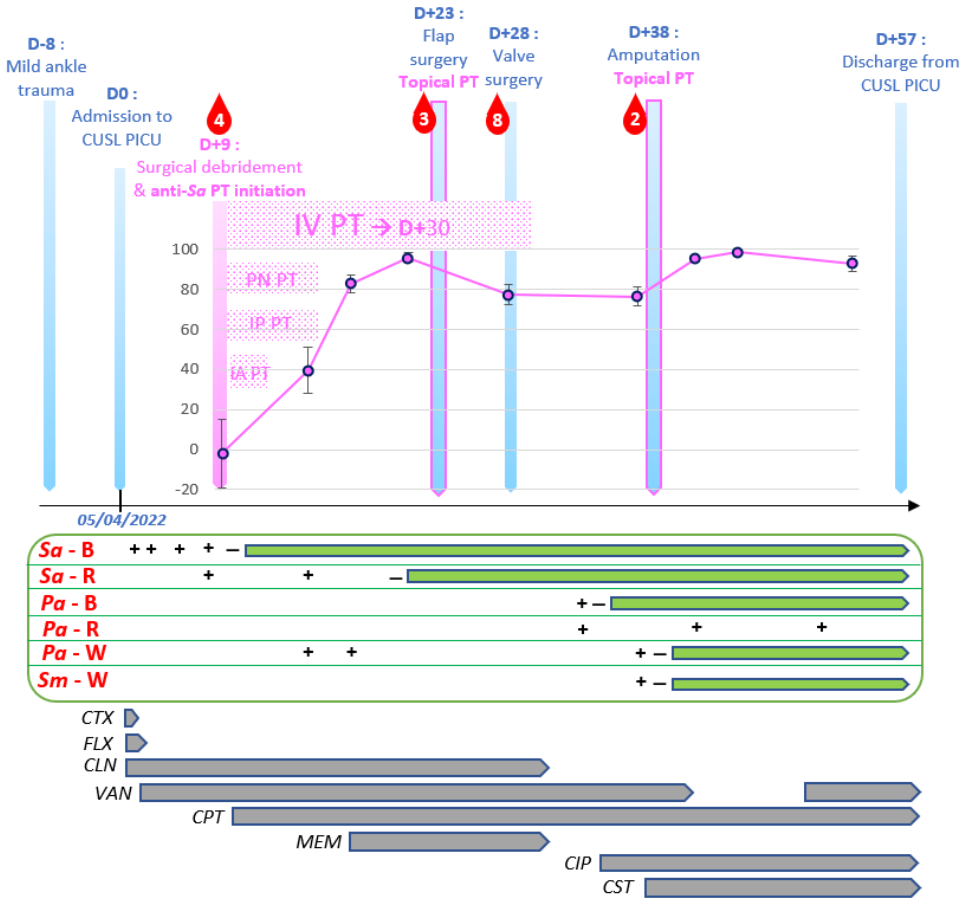


Fig. III.12 | Case 3 – Timeline of the clinical case's main events. Main medico-surgical events are represented as blue vertical arrows. Phage therapy-related events are represented in pink. Pink curve represents degree of Phage Immune Neutralization (PIN) against phage ISP in % of neutralized titer at 30 minutes. Error bars represent standard deviation of the means. Red droplets represent units (205 mL) of blood transfused. D – day ; *Sa* – *Staphylococcus aureus* ; *Pa* – *Pseudomonas aeruginosa* ; *[…]*

[…] Sm – Stenotrophomonas maltophilia ; B – Blood culture ; R – Respiratory tract-originated sample culture (broncho-alveolar lavage or endotracheal aspirate) ; W – wound sample culture (swab or intraoperative biopsy) ; PT – phage therapy ; IV – intravenous ; PN – pulmonary nebulization ; IP – intra-pleural ; IA – intra-abdominal ; CTX – Cefotaxime ; FLX – Flucloxacillin ; CLN – Clindamycin ; VAN – Vancomycin ; CPT – Ceftaroline ; MEM – Meropenem ; CIP – Ciprofloxacin ; CST – Colistin

A 12-year-old boy with no significant medical or surgical history was transferred (D0) to the Pediatric Intensive Care Unit (PICU) **(Fig. III.12)**. On D-8, he suffered a minor trauma causing a sprained left ankle with superficial scratches. A plaster boot cast was set on D-7, but removed on D-6 because the patient reported increasing pain. On D-3, the child became confused and on D-1, febrile at 39°C. Clinical findings included tachycardia, polypnea, and swollen, painful, warm and red left leg and foot. Biological findings included signs of inflammatory syndrome (C-reactive protein 304 mg/L - normal value <5 mg/L) and multiple organ failure including respiratory and hemodynamic failure as well as liver, kidney and muscle damage, leading to PICU admission. Blood cultures were performed and empirical IV flucloxacillin, clindamycin and cefotaxime were initiated. Exceeding intramuscular pressure was measured in all eight tested compartments in the left foot and leg. Fasciotomies were performed, revealing pus in the soft tissues of the left lower limb.

Vasopressor therapy, sedation and endotracheal intubation were maintained upon return to PICU. Microbiological cultures of pus and blood

grew for MRSA producing Panton-Valentine Leukocidin (PVL). IV vancomycin was initiated on D+1, replacing cefotaxime and flucloxacillin, while clindamycin was pursued. Since we suspected significant contribution from the PVL toxin to the patient's condition, IV polyclonal immunoglobulins (1 g/kg) were administered on D+1.

Despite these adapted SOC measures and in-range therapeutic drug monitoring of vancomycin within 48h of initiation, the infection rapidly progressed to generalized necrotizing fasciitis and pyomyositis on D+2, as confirmed by full-body MRI. This justified extended fasciectomies and debridement on multiple sites on D+8. Ascites appeared and was drained on D+2. Thoracic CT-scan revealed disseminated septic emboli in both lungs on D+1. Chest X-ray showed a right pleural effusion on D+8, which was drained with purulent discharge confirming empyema. Microbiological cultures of both the peritoneal and pleural fluids grew for the same MRSA-PVL strain.

Between D0 and D+8, sustained MRSA-PVL bacteremia was observed and the patient's state was significantly deteriorating to a life-threatening situation, despite maximal SOC management.

As rescue therapy, PT was initiated on D+9. A solution containing the anti-*S. aureus* phage named ISP was obtained from Queen Astrid Military Hospital ([QAMH], Brussels, Belgium) after on-plate phage susceptibility testing ("phagogram") revealed that phage ISP had potent lytic power against the patient's MRSA-PVL strain (EOP = 1, i.e. "efficiency of plating", a phage's relative lytic efficiency on a clinical strain compared to the phage's

reference host strain, expressed as a ratio of the number of plaques theoretically ranging from 0 to 1). The initial ISP phage solution, of titer 10^9 PFU/mL, was diluted 100 times in NaCl 0,9% to reach a therapeutic titer of 10^7 PFU/mL. Multi-route PT was then initiated using this 10^7 PFU/mL solution **(Fig. III.12)**. IV PT was administered at 50mL over 6h every 24h during 22 days (D+9 to D+30). Intra-pleural and intra-peritoneal PT were administered through the respective drainage catheters: once daily, cavities were rinsed with 5% sodium bicarbonate solution for 15 minutes, before 40mL of phage solution were instilled. Catheters were then clamped for 20 minutes before being reopened, without aspiration. Topical PT was applied daily on open wounds with dressings soaked in phage solution for 20 minutes. Lastly, bronchopulmonary PT was administered by mesh-nebulization of 4 mL of phage solution at the higher titer of 10^8 PFU/mL. IV ceftaroline was also added to the treatment eight hours after the first IV phage dose. Additionally, following isolation of *Pseudomonas aeruginosa* in several wounds on D+14, IV meropenem was administered from D+17 until D+30 **(Fig. III.12)**.

Objective improvements were rapidly noticed. The first blood culture performed after PT initiation (on D+10, 30h after PT initiation and 22h after ceftaroline initiation) did not grow for MRSA-PVL, nor did any ulterior blood culture. Peritoneal and pleural catheters were withdrawn after three and seven days of PT respectively. Despite these improvements, the evolution remained complicated, with extubation failure related to persistent lung damage and bacterial tracheitis, and mitral valve abscess related to sustained MRSA-PVL bacteremia, cured by open-heart valve repair surgery on D+28. Septic necrosis led to extensive tissue loss in the left foot and leg, with no hope for

spontaneous wound closure. Reconstruction by flap surgery using right latissimus dorsi was attempted on D+23. Intra-operative topical PT was applied using ISP solution (10^7 PFU/mL) for syringe rinsing of the surgical site and soaking of dressings applied for 45 minutes. However, flap necrosis started on D+32, eventually leading to transtibial amputation of the left leg on D+38. Infection of the left leg wounds by *Stenotrophomonas maltophilia* and *P. aeruginosa* had been diagnosed on D+37. Accordingly, we administered intra-operative topical PT on the leg stump before surgical closure, applying soaked dressings and rinsing with 200 mL of a 10^7 PFU/mL mix of anti-*S. maltophilia* phage BUCT700 and anti-*P. aeruginosa* phages PNM, 14-1 and PT07.

After amputation, sustained favorable clinical progression was observed along durable eradication of all *S. aureus*, *S. maltophilia* and *P. aeruginosa* from the bloodstream and all wounds. Besides amputation, the child progressively reached full recovery of all wounds and of respiratory function. The child was transferred from PICU to pediatric hospitalization unit on D+57, then to rehabilitation unit on D+104. Final discharge from hospital occurred on D+145. Two years after hospital discharge, the patient remains cured of all infections. Stump osteitis was suspected on clinical basis 5 months after hospital discharge, and was successfully treated by surgical revision along with vancomycin and clindamycin administration. Functional rehabilitation and adaptation to the prosthetic leg appear successful. Cardiological follow-up upon initial hospital discharge diagnosed ectopic atrial tachycardia, likely linked to previous bacterial valve abscess and related surgery. This was initially treated by flecainide, which was successfully withdrawn after 4 months without needing further re-introduction.

In conclusion, this severe infection required an exceptional multiple bacteriophage-antibiotic combination therapy directed against three pathogens: *S. aureus*, *P. aeruginosa* and *S. maltophilia*. This is likely the first report ever of a patient being treated by three distinct documented phage therapies, targeting three distinct pathogens, all with their dedicated route(s) of administration and combined antibiotic therapy. Sustained MRSA bacteremia and its associated metastatic sites of infection are a known therapeutic challenge: seemingly appropriate SOC management often fails to eradicate bacteremia, and PT is increasingly reported as a potentially promising complementary approach[188]. In this case, PT initiation was rapidly followed by the eradication, in the treated compartments, of these three pathogens. Yet, several aspects of the clinical and microbiological progression deserved further attention.

The occurrence of PIN, the development of an antibody response against administered phages, was again investigated using modified Adams protocol by double agar overlay assay on numerous serum samples collected before, during and after PT[168]. PIN occurred against phage ISP, starting six days after PT initiation (D+14) **(Fig. III.12)**. Transient decrease in PIN magnitude between D+21 and D+41 is likely due to hemodilution by numerous blood transfusions **(Fig. III.12)** and extracorporeal circulation during open-heart surgery. Consistent with previous findings, this potent and early-onset PIN was not followed by therapeutic failure, though it might mitigate the efficacy of ulterior reintroduction of phage ISP[128, 148].

Evaluation of PT's net contribution to *S. aureus'* eradication from the bloodstream is a limitation to this work. It is indeed difficult because of the

initiation of ceftaroline eight hours after PT initiation and the absence of blood cultures that could have been collected in this short interval between PT initiation and ceftaroline initiation.

III.3 Case-based observations and conclusions

As previously mentioned, the heterogenicity of our seven cases across a number of demographical, microbiological, clinical or therapeutic criteria makes it hard to derive any specific and robust conclusion from them. Here, we will not re-review in full the pre-clinical and clinical literature that was already discussed in section II.4. However, we will attempt to formulate a number of synthetic observations, based on this admittedly limited clinical experience. Some tentative recommendations and remaining "grey areas" will also be pointed out.

To complete the descriptive characteristics of these seven cases, presented above in **Table III.2**, further outcome-related characteristics are depicted in **Table III.3**.

Case #	Age (years)	Sex	Clinical indication	PT-targeted pathogen(s)	AMR profile	Used phage(s)	Phage susceptibility	PT route(s)	AB attempted prior to PT	AB during PT
1	13	F	Chronic pelvic osteomyelitis	***S. aureus***	iMLSB MSSA	ISP	Spot test +	Intra-operative lavage, *in loco* instillations	Clindamycin, rifampin, flucloxacillin	Clindamycin, rifampin, ciprofloxacin
2	1	M	Bacteremia, intra-abdominal infection	***P. aeruginosa***	XDR VIM-2	PNM, 14-1, ISP	PNM EOP = 0.3 Others : 0	Intra-operative lavage, intravenous, biliary instillations	Colistin, gentamycin, aztreonam	Same as prior to PT
3	12	M	Bacteremia, necrotizing fasciitis, arthirtis, pneumonia, intra-abdominal	***S. aureus*** ***P. aeruginosa*** ***S. maltophilia***	MRSA MDR WT	ISP PNM, 14-1, PT07 BUCT700	ISP EOP = 1 Others : spot test +	Intra-operative lavages, intravenous, intra-abdominal & intra-thoracic instillations, pulmonary	For MRSA-mediated necrotizing fasciits and associated bacteremia : vancomycin, clindamycin	Vancomycin, clindamycin, ceftaroline, meropenem, ciprofloxacin, colistin
4	43	F	Intra-abdominal infection	***E. faecium***	VRE vanA	KN	Spot test +	Intravenous, intra-abdominal instillations	Multiple courses of Linezolid	Linezolid, Vancomycin
5	1.5	F	Necrotizing cellulitis	***P. aeruginosa***	WT	C, 4P	Spot test +	Intra-operative lavage, topical	Piperacillin-tazobactam, amikacin	Same as prior to PT
6	17	M	Sub-cutaneous surgical scar infection	***S. aureus***	WT (MSSA)	ISP	Spot test +	Intra-operative lavage, *in loco* instillations	Multiple attempts : oral flucloxacillin, doxycyclin, clindamycin, ciprofloxacin, cotrimoxazole	Clindamycin, cefazolin
7	70	M	Chronic femoral osteomyelitis	***P. aeruginosa***	WT	DP1, 4P	EOP = 0.01 each	Intra-medullary instillations	Multiple attempts : meropenem, ceftazidime, piperacillin-tazobactam, ciprofloxacin	Ceftazidim

Case #	Age (years)	Sex	Clinical indication	PT-targeted pathogen(s)	Additional measures	Final clinical response	Micro-biological response	PIN development	PAI(/PAS) evaluation	BPR evidence	Stacey et al. efficacy criteria met	Intra-operative PT	Full coverage of infectious species by PT	PT hoping to avoid major surgery
1	13	F	Chronic pelvic osteomyelitis	***S. aureus***	Surgical revision with intra-operative phage lavage	relapse	no	not evaluated	after PT	none	yes	yes	no	yes
2	1	M	Bacteremia, intra-abdominal infection	***P. aeruginosa***	Liver re-transplantation with intra-operative phage peritoneal lavage	full resolution	yes	developed for PNM only	after PT	yes	yes	yes	yes	no
3	12	M	Bacteremia, necrotizing fasciitis, arthirtis, pneumonia, intra-abdominal	***S. aureus*** ***P. aeruginosa*** ***S. maltophilia***	Left trans-tibial amputation, multiple soft tissue and articular debridement, thoracic and peritoneal drainage	full resolution	yes	developed for ISP	after PT	none	yes	yes	yes	no
4	43	F	Intra-abdominal infection	***E. faecium***	Pre- and post-phage therapy surgical revision without intra-operative phage lavage	partial resolution	yes	not yet evaluated	before PT	none	yes	yes	yes	no
5	1.5	F	Necrotizing cellulitis	***P. aeruginosa***	Surgical revision with intra-operative phage lavage, abscess drainage	full resolution	yes	not yet evaluated	not yet evaluated	none	yes	yes	yes	no
6	17	M	Sub-cutaneous surgical scar infection	***S. aureus***	Surgical revision with intra-operative phage lavage	partial resolution	yes	not yet evaluated	not yet evaluated	none	yes	yes	yes	yes
7	70	M	Chronic femoral osteomyelitis	***P. aeruginosa***	Surgical revision without intra-operative phage lavage	relapse	no	not evaluated	not yet evaluated	not yet evaluated	yes	no	yes	yes

Table III.3 (preceded by Table III.2 – reprise) | Main outcome-oriented characteristics of the 7 phage therapy cases. "Stacey et al. efficacy criteria" refers to the abovementioned criteria proposed in a phage therapy trial review by Stacey and colleagues in 2022: essentially the delivery of a sufficient amount of efficacious phages, at the right site of infection, to target a sufficient amount of susceptible infectious bacterial cells[189]. PT, phage therapy; AB, antibiotic; WT, wild type; AMR, antimicrobial resistance; iMLSB, inducible macrolides-lincosamides-streptogramin B; MRSA/MSSA, methicillin-resistant/susceptible *S. aureus*; MDR, multidrug resistant; XDR, extensively drug resistant; VIM-2, Verona integron-encoded metallo-beta-lactamase type 2; VRE, vancomycin-resistant *Enterococcus*; PIN, phage immune neutralization; PAI, phage-antibiotic interaction(s); PAS, phage-antibiotic synergy.

An interesting conclusion from our series is that in most of these seven cases, literal *bacterial* resistance to antibiotics was not the primary motive to consider PT. Instead, most indications represented a therapeutic dead-end of a resistant *infection* whose resistance to SOC treatment could not or not fully be attributed to *bacterial* AMR. This is undoubtedly the case for cases 1, 5, 6 and 7 in which incriminated bacterial isolates exhibit little to no clinically-significant AMR, but are however characterized by infections likely localized in territories of poor antibiotic penetration. Case 1 and case 6, for example, are both ex-Ewing sarcoma patients, suffering from infections occurring at or around a bone allograft, a tissue of suboptimal vascularization, a phenomenon even worsened by the implantation of these grafts in previous sites of

radiation therapy[190]. As previously discussed, such irradiation likely mitigates antibiotic penetration in these locations and might represent a hurdle in the treatment of both skin-and-soft-tissue infections and bone-and-joint infections[169, 170]. In these cases, the poor antibiotic response of these infections, and the subsequent need for PT, are thus probably more associated to these factors than to bacterial AMR, which is respectively minor (iMLSB MSSA) and absent (wild-type MSSA) in these two cases. Among these two, chronic biofilm organization was an additional hurdle in case 1 and was also thought to be the main reason of antibiotic failure in case 7, a patient who had suffered from a chronic post-traumatic osteomyelitis caused by wild-type *P. aeruginosa* for 45 years[191]. Lastly, a third factor of poor antibiotic penetration and incurred therapeutic failure is necrosis, thought to be a prominent factor in antibiotic failure in already discussed case 3, but also in case 5[192].

Contrastingly, infections that were refractory mainly due to bacterial AMR, probably thought to be the most common PT indications by the general public, were a minority in this series: case 2 and arguably cases 3 and 4 fit into this category.

Five out of these seven cases were associated with durable post-PT microbiological eradication of the targeted bacterial species, and the same five cases were also associated with at least partial remission of clinical manifestations, three of them being considered in full clinical remission. Case 4 was considered a partial clinical remission as she later manifested new signs of intra-abdominal inflammation and digestive transit dysfunction, though these were considered less intense than before and were not associated with subsequent isolation of new *E. faecium* isolates (but rather other bacterial

species such as *K. pneumoniae*). She later benefited from subsequent digestive surgical revision on that regard, which was associated with good clinical response. Case 6 was also considered a partial clinical remission: he was initially treated for superficial surgical scar infection nearing a femoral bone allograft implanted in an irradiated area. The scar presented a pseudo-cystic bulging with a cutaneous fistula discharging pus on a daily basis. This bulging was attenuated but not fully suppressed after PT and clinical revision. The fluid effusion still occasionally occurred but on significantly rarer occasions, and was less purulent on a visual basis. Microbiological culture of this fluid did not grow for *S. aureus* again. All in all, the clinical inflammatory aspects of the treated area were all considered significantly improved.

Distinguishing clinical from microbiological response in PT cases description remains important. Indeed, even though we observe a correlation between microbiological eradication and clinical improvement in our series, we should note that:

- Two out of five cases presenting microbiological eradication did not, however, present full clinical resolution of the initial presentation. This observation leads to a critical question: was the initial clinical problematic entirely attributable to the refractory infection? Which itself calls for additional questions: what could reasonably be hoped for after bacterial eradication? What was done to simultaneously address the "non-infection-related" aspects of the problematic? In our experience, the assessment of these questions prior to considering PT is critical, and probably of underrated importance. Underestimating this part of the pre-therapeutic assessment, and

the transparent patient communication it calls for, can lead to unrealistic hopes and subsequent disappointments in both physicians and patients.

- Conversely, and perhaps more surprisingly, decorrelations between microbiological eradication and clinical improvement have also been observed the other way around. Indeed, recent literature has described situations in which post-PT clinical improvement was observed, even though isolates of the targeted bacterial species could still be retrieved in the treated compartment. In 2023, Köhler and colleagues reported such a case in which pulmonary nebulization of anti-*P. aeruginosa* phages was attempted to treat chronic MDR-*P. aeruginosa* lung infection in a 41-year old man suffering from Kartagener syndrome[193]. It was likely the first case report to illustrate human in-lung replication of phages administered this way. However, such an active phage replication did not manage to eradicate the *P. aeruginosa* presence in the lung, post-PT isolates nevertheless remaining phage-susceptible. Still, post-PT period was marked by objective and sustained (> 1 year) clinical improvement (decrease in dyspnea and sputum production, ability to resume professional activity) as well as radiological improvement (decrease of pathological findings in thoracic CT-scan). Whether such a microbiological-clinical decorrelation could be explained by PIVT was investigated by Köhler and colleagues: however, no result from either Calu-3 cell cultures nor *G. mellonella* assays indicated virulence loss in post-PT *P. aeruginosa* isolates. As is, the mechanistic basis of such fortunate clinical outcome thus remains elusive.

Besides the post-hoc analysis of such cases' outcomes, however, the identification of robust pre-, per- and/or post-therapeutic criteria to predict

therapeutic failure or success appears as a major challenge for future PT clinical research. Finding such common traits reliably discriminating and ideally predicting therapeutic failures has been attempted. As already discussed in section II.4, at the clinical trial level, Stacey and colleagues have recently proposed a simple list of criteria that were systematically unmet in trials showing disappointing efficacy outcomes[189]. These criteria can essentially be boiled down to "the right amount of the right phages, delivered at the right place, at the right time during infection progression, to target a sufficient number of bacterial cells". However, as depicted in **Table III.3**, all of our seven cases seem to match these criteria, including cases 1 and 7 whose microbiological and clinical evolutions have turned out unsatisfactory. Though these criteria have not been proposed for single case analysis but for trial analysis, this mismatch still leads us to postulate that Stacey and colleagues' criteria might fit a "necessary but insufficient" scenario, or that some of them could benefit from a more precise definition.

In that regard, PT failure in case 7, for example, still escapes obvious explanation. We suspect the lack of initial intra-operative phage lavage to be a key limitation, potentially preventing the most efficient direct access of phages to the whole infected site compared to subsequent intra-medullary instillations. Low EOP (0.01) for both used phages while still retaining a therapeutic titer of 10^7 PFU/mL may have led to borderline effective phage titers. Lastly, antagonistic phage-antibiotic interactions with ceftazidime, administered before and during PT, was not yet excluded. As this relapse is very recent, BPR emergence has not yet been fully assessed. Similarly, PT failure in case 1, as already explained, cannot be technically attributed to

unmet Stacey efficacy criteria, but most likely rather to the partial coverage of the multi-bacterial infection by PT.

Ultimately, these efficacy criteria, while still partly to be defined, can be translated in a tentative list of "do's and don'ts" regarding both case selection and management. Such a task has been recently attempted by Suh and colleagues in their extensive 2022 recommendations for good clinical practice in PT[108]. To conclude, taking inspiration from the recommendations and rationales detailed in this work and based on our additional experience, we propose the following :

- Case selection:

- "Green flags" – suggestive of a good PT indication: use of PT for a clinical indication that has already been reported as successful in literature, ideally on multiple occasions; availability of at least one phage showing significant efficacy on the targeted bacterial strain through an already reported phage susceptibility testing method; clear documentation that the considered infection is refractory despite SOC treatment attempts including antibiotics and needed complementary therapeutic approaches (e.g. surgical revision, collection drainage); clear documentation of the extension of the infection by relevant medical imaging and/or intra-operative findings, for example; possibility to administer PT to all these infected compartments by relevant administration routes; in the case of multi-bacterial infections, clear documentation that all bacterial species deemed responsible for this refractory infection have been identified and can all be targeted by PT; pre-therapeutic assessment that both the treating physician(s) and the patient are willing to endure complementary therapeutic approaches to PT if they are

considered necessary (e.g. surgical revision, collection drainage), and are not considering PT as a way to avoid them.

- **"Grey areas" -** should ideally be avoided, and either clarified by future research and/or attempted with caution and reasonable expectations: partial therapeutic overage of a multi-bacterial infection; intra-cellular infections; use of phages whose phage susceptibility testing results show suboptimal efficacy (e.g. EOP <0.01) or inconsistency between testing modalities, including phages likely to exert only "lysis from without" effect[194]; considering PT for a clinical indication that does not belong to the "Red flags" category but that has never been described in literature before.

- **"Red flags"** – avoid considering PT: absence of evidence of active infection caused by the treated pathogen (unless specifically aiming at carriage decolonization) ; clearly disproportionate hope in PT as a way to avoid a major surgery that would appear mandatory even in the absence of active infection (e.g. amputation of a limb); PT as first line anti-infective treatment prior to SOC antibiotic therapy; inability to ensure an adapted administration route (e.g. treating a bacteremia without the will or possibility to administer intravenous PT).

Case management (once case is selected with the help of the above):

- **"Do's":** plan PT so that it fulfills aforementioned Stacey and colleagues "efficacy criteria"; on that regard, use relevant administration route(s) and phage titers in accordance to previously-reported similar cases and/or seek expert opinion if unsure; collect pre-therapeutic blood sample

assessing at least baseline hemogram and both renal and liver functions, and monitor these biological parameters at least once a week for the whole duration of PT; collect serum samples in a similar fashion to assess PIN reaction, even if it is only evaluated post-hoc; always maintain antibiotic therapy along with PT; to guide antibiotic co-treatment selection, perform phage-antibiotic interactions testing ideally before initiating PT, especially focusing on antibiotics that would be considered clinically relevant in the situation and using an already-reported method; if this is not realizable, still maintain antibiotic therapy during PT, even if it is with antibiotics that have been considered unhelpful in the earlier management of the case; perform thorough intra-operative phage lavage when applicable; in the case of intravenous administration, maintain close clinical surveillance of the patient especially during the first intravenous administration; collect relevant biological samples during and after PT to potentially culture new bacterial isolates (and screen them through phage susceptibility testing again) and phage isolates (as pharmacokinetics documentation and possibly for co-evolution studies); document objective ways of assessing both clinical and microbiological progression during and after PT; if you suspect any adverse event during PT, even if you do not firmly believe it is caused by PT, document and notify it to relevant authorities and seek advice from experts.

- **"Don'ts":** do not withdraw antibiotic therapy during PT; do not withdraw PT when suspecting BPR acquisition in a bacterial isolate during PT; do not withdraw PT when suspecting PIN reaction development; do not withdraw PT because some biological samples collected after PT initiation still grow for the targeted pathogen; do not automatically assume that a given clinical or biological reaction happening during or after PT is

likely not an adverse event; avoid publication bias inclined favorably towards successful PT cases.

- "Grey areas" – the interest of these complementary measures has theoretical basis but should be clarified in further clinical research: in patients not already treated by antibiotics at the time when PT is considered, consider the sequential introduction of PT shortly before antibiotic co-treatment ("phage first" approach); avoid using phage-antibiotic combinations that you found out to be antagonistic or that have already been described as antagonistic in literature if another non-antagonistic antibiotic is considered equally relevant.

Chapter IV: Liver transplantation and associated bacterial infections

–

The OPTICS study

IV.1 Introduction

Liver transplantation (LT), the act of surgically replacing a patient's liver with the entirety or a part of another human liver, can be considered the most radical curative treatment option for a number of clinical indications involving hepatocellular dysfunction. Since it was first performed in 1963 by Thomas Starzl, both its safety and efficacy outcomes have dramatically improved thanks to multifactorial progress in both medical and surgical management[195, 196]. Despite the emergence of living donor liver transplantation (LDLT) to address some LT indications in complement with traditional deceased donor liver transplantation (DDLT), organ shortage likely remains the most restrictive limitation to the procedure[195, 196]. This implies a careful selection of the best indications of LT, to ensure each LT carries a satisfactory risk-benefit ratio. The list of such validated indications has been subject to regular changes, and includes both malignant and non-malignant conditions, among which numerous conditions inducing chronic liver disease up to a stage of liver cirrhosis (e.g. congenital or infancy-acquired bile duct diseases such as biliary atresia, conditions related to

exogenous agents exposure such as alcoholic or non-alcoholic (fatty) liver disease, auto-immune conditions such as primary biliary cirrhosis, genetic conditions such as alpha-1 antitrypsin deficiency), or some cases of primary liver neoplasms such as hepatocellular carcinoma[196].

However, despite its potential for life-changing curative outcomes and quality of life improvement, LT is no trivial intervention and its associated morbidity and mortality remain significant to this day[197]. These are related to both numerous short- and long-term medical and surgical complications[198, 199]. Short-term complications include peri-operative technical complications affecting the graft parenchyma itself or its related blood vessels and biliary system (e.g. surgical injury, hemorrhage, thrombosis, fistula), early medical complications related to the major surgery and the modifications brought by the liver transplant on the global physiology of the recipient (such as hemodynamic instability or electrolytic alterations for example), and medical complications related to the implanted liver function and viability such as hyperacute and acute graft rejection[198, 199, 200, 201, 202]. All of these are susceptible to lead to early liver graft dysfunction. Long-term complications include so-called chronic rejection, complications associated with anti-rejection drugs side effects, and broader multifactorial alterations to the physiology of the recipient such as an increased tendency to develop arterial hypertension, chronic renal failure, dyslipidemia or specific cancers like non-melanoma skin cancer or lymphoma for example[198, 203, 204].

Nevertheless, infections are widely considered the most prominent factor of short-term post-operative morbidity and mortality in liver transplantation recipients[131, 205, 206, 207, 208]. Typical post-LT infections are

varied and include bacterial infections, sometimes caused by nosocomial opportunistic pathogens during early post-LT hospital stay, but also various viral (e.g. caused by Epstein-Barr virus, cytomegalovirus, herpes simplex virus, human herpes virus subtypes 6 and 7), fungal (e.g. caused by *Candida* spp, *Aspergillus* spp., or more rarely *Pneumocystis jirovecii*) and parasitic infections (e.g. caused by *Toxoplasma gondii*) ; among them, though, bacterial infections are especially frequent in the few weeks to months following LT[131, 205, 207]. These typically include bloodstream infections, intra-abdominal infections and surgical site infections for example[131].

The risk to suffer from one of these post-LT bacterial infections likely decreases over time and appears to be especially high during the first one or two post-LT month(s) **(Fig. IV.1)**[131, 207, 208]. Among these, the earliest infections developing in the first post-LT month are typically related to the surgical procedure itself and possible incurred medical complications: this is not dissimilar to the infectious risk caused by other major abdominal surgeries, notably those involving the biliary system, but the procedural complexity and length of the surgical procedure, the underlying medical condition and the immunosuppressive medication involved represent additional risk factors explaining a specifically higher infectious incidence in LT recipients[207]. Bacterial infections happening during the two to six post-LT months period are less frequent, paralleled by an opposite trend of increased viral infection incidence, and are thought to correlate more importantly to individual patient-related risk factors and immunosuppressive medication overexposure than to the surgical procedure itself, though the necessity for post-LT surgical revisions notably increases this risk[131, 207]. A number of

clinical research efforts have attempted to list these numerous surgical- and patient-related risk factors **(Table IV.1)**[205, 207, 209].

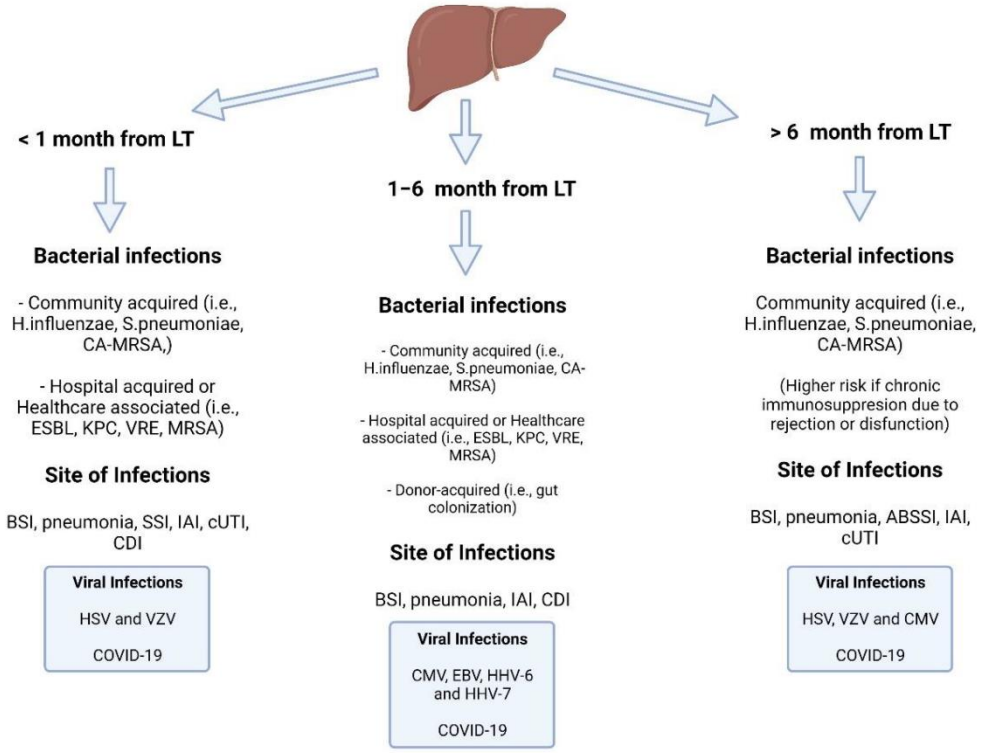


Fig. IV.1 | Timeline of bacterial and viral infections in the liver transplant (LT) recipient. *CA-MRSA, community-acquired MRSA; ESBL, extended-spectrum beta-lactamase; KPC, Klebsiella pneumoniae carbapenemase; VRE, vancomycin-resistant Enterococcus; BSI, bloodstream infection; SSI, surgical site infection: IAI, intra-abdominal infection; cUTI, complicated urinary tract infection; CDI, Clostridium difficile infection; HSV, herpes simplex virus; VZV, varicella zoster virus; EBV, Epstein-Barr virus; HHV, human herpes virus; ABSSI, acute bacterial skin and soft tissue infection.* From Shbaklo et al., 2022[208].

Transplant factors	*Recipient*
Ischaemia times, ischaemia-reperfusion damage	Underlying condition of the recipient, i.e. malnutrition
Infected preservation fluid	Co-morbidity, e.g. diabetes, obesity, COPD, renal failure and dialysis
Amount of intra-operative blood transfusion	Colonisation with *S. aureus* or resistant organisms
Level and type of immunosuppression (e.g. anti-CD25)	Prolonged hospital stay and catheters before OLT
Additional immunosuppression for rejection	Acute liver failure
Indwelling catheters, deep lines	CMV-status and disease (risk for other infections)
Complications like primary non-function, hepatic artery thrombosis, necrosis, biliary strictures	Presence of hepatitis B or C virus or HIV
Prolonged ICU-stay, dialysis, prolonged ventilation	MELD score >30
Type of biliary drainage (Roux-en-Y, T-tube)	Recipient age
Repeat operations and re-transplantation	Previous immunosuppression (autoimmune hepatitis; re-transplantation)
Antibiotic regimen	Previous infection (esp. airway, urine tract)
Viral prophylaxis and monitoring	Immune status for viruses
Environment (other infected patients, building activity, hygienic measures)	Male recipient receiving male donor liver
	Hygienic measures
	Travelling
Genetic polymorphisms in innate immunity	*Donor*
Lectin pathway of complement activation in donor and donor/recipient mismatch (MBL2, ficolin2, MASP2)	Infection in donor
Toll-like receptors in the recipient	Prolonged ICU-stay
	Quality of the liver graft (e.g. marginal graft)
	Viral status

Table IV.1 | Risk factor for infections after liver transplantation. From van Hoek et al., 2012. OLT, orthotopic liver transplantation; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; CMV, cytomegalovirus; MELD, Model for End-stage Liver Disease

Another risk factor could be that the asymptomatic digestive carriage of specific bacterial strains in pre-LT recipients might lead to development of actual post-LT infections caused by these strains. In this paradigm, post-LT infection is the result of a "carriage-infection conversion" (CIC) mechanism in which the LT acts as a trigger event: the rationale for this can be related to the aforementioned surgical heaviness of the procedure, its involvement of the digestive tract itself (especially when Roux-en-Y anastomosis is preferred to duct-to-duct anastomosis, which is classically the case in pediatric LT), and the incurred immune suppression[210, 211]. In addition, a related concept to this paradigm would be that eradicating this pre-LT carrier status could be able to mitigate its associated post-LT infectious risk, allowing for a preventive approach. This concept has been the subject of clinical and research interest for at least the last four decades[212]. In 1987 indeed, Wiesner and colleagues performed "selective bowel decontamination" (SBD) in 30 pre-LT patients using broad spectrum non-absorbable oral antibiotics and antimycotics, and suggested that the marked reduction in Gram negative bacteria and *Candida* spp. probably led to a lesser post-LT early infection rate[213]. A larger series of cases submitted to a similar protocol was published six years later by Steffen and colleagues, though still a key limitation in the absence of a prospective control group[214]. Since then, numerous works have addressed varied aspects of this problematic. Notably, in 2012, Bert and colleagues published a single-center series of over 700 LT recipients from France, advocating for an independent infectious risk caused by pre-LT carriage of ESBL-Enterobacterales, going as far as illustrating that the post-LT infections caused by these ESBL-Enterobacterales were mainly attributable to this CIC mechanism ("endogenous" infections) and not to "exogenous" post-LT

nosocomial contamination of patients that were no pre-LT carriers[215]. Several works from the same era have illustrated that post-LT infections caused by such MDR/XDR bacteria were associated to a specific increase in morbidity and mortality[216, 217]. The seemingly robust correlation between pre-LT MDR bacteria (MDRB) digestive carriage and post-LT infections caused by these pathogens has been reported again in more recent works, including in the pediatric population[218, 219].

Shortly after their aforementioned series, Bert and colleagues published the results from another ulterior, smaller cohort of 317 patients, noting as a new point of interest that previous exposition to aforementioned SBD was one of the risk factors predicting for ESBL-Enterobacterales digestive carriage[220]. Other works have supported the hypothesis that SBD could induce secondary antimicrobial resistance, like in the case of oral colistin and gentamycin use for carbapenem-resistant *K. pneumoniae* decolonization[221].

These respective aspects of the problematic can be combined into three closely related conclusions, summed up by Catho and Huttner in their 2019 review and expert opinion, for example [222]:

- **CIC concept:** MDRB carriage status is correlated to the development of proper infections caused by these pathogens, the magnitude of this risk varying between series.

- **CIC risk factors:** risk factors proposed for the triggering of such carriage-infection conversions include intensive care stay, major abdominal

surgery, and immunosuppression, all three of which are systematically present in liver transplantation recipients.

- CIC prevention / management: interventional strategies aiming at eradicating the initial MDRB carriage, in an attempt to prevent CIC, have proven disappointing. These strategies include oral antibiotics regimens as the most traditional decolonization strategy, inspired by abovementioned historical SBD protocols, but also more recently described strategies based on oral probiotics or fecal microbiota transplantation (FMT). The most recent and comprehensive work discussing the modalities and efficacy of these strategies, and confirming Catho & Huttner conclusions, is probably the 2023 systematic review of Mascolo and colleagues regarding decolonization of MDR Enterobacterales digestive carriages[223]. Out of 679 initially screened records, Mascolo and colleagues performed the analysis of 12 clinical studies, among which 4 were RCTs. Two of them evaluated FMT, two of them evaluated oral probiotics (one of which was in combination with oral antibiotics), and the remaining eight evaluated various regimens of oral antibiotics. Mascolo and colleagues noted that the analysis of these works suffered from small sample sizes and largely heterogenous definitions of effectiveness and treatment duration, for example. Nevertheless, similar to Catho and Huttner, they conclude that the current body of evidence does not advocate for reliable, significant efficacy of these decolonization strategies, and that these should not be used outside of a clinical research perspective before ulterior, more standardized works would potentially revoke this recommendation. A 2022 review by Karbalaei and colleagues specifically addressing oral probiotics strategies efficacy in MDRB digestive decolonization came to the same conclusions[224]. Lastly, the specific analysis

of FMT efficacy potential in this indication appears as more elusive and conflicting, given that it is likely the most recently introduced - and lesser studied - decolonization strategy among these three strategies to this date. The reference review work addressing this question is likely the 2022 review by Bilsen and colleagues[225]. The authors summarize the current level of evidence by concluding that since 2020, only seven non-controlled, small sized studies have evaluated FMT's efficacy in MDRO decolonization following vastly heterogenous protocols and generating conflicting outcome results with decolonization rates ranging from 20 to 90%. They acknowledge the fact that these potentially promising results absolutely warrant further standardization of the FMT protocols, efficacy assessment and bias mitigation (regarding spontaneous decolonization rates, *inter alia*) before considering its routine clinical use in this indication. Recent works have also documented that FMT, while generally considered safe, could on rare occasion lead to severe and even lethal adverse events, for example when unknowingly transplanting feces containing MDR Enterobacterales to an immunocompromised patient, leading to iatrogenic infection by translocation[226].

This lack of validated solution for an important clinical problematic leaves room for additional, yet to be tested strategies to mitigate the burden of CIC. The use of oral PT has been mentioned among them by Catho & Huttner, a concept that they however qualified as "science fiction" in the 2019 current state of knowledge: this provided the conceptual basis for the OPTICS study that we carried out, and that will now be detailed[222].

IV.2 The OPTICS study: design

Pediatric LT is an especially important clinical activity in our clinical setting (Cliniques universitaires Saint-Luc [CUSL], Brussels, Belgium), and is the clinical population on which was focused the clinical research presented in this chapter. CUSL are indeed one of the world's reference centers for pediatric LT, mainly for historical reasons. Indeed, Jean-Bernard Otte and Paul-Jacques Kestens, two retired CUSL surgeons, were early pioneers of pediatric LT: they were among the first to successfully perform it in 1971, four years after the first pediatric LT ever performed in 1967 by aforementioned Thomas Starzl (who taught Jean-Bernard Otte between 1965 and 1966), and three years after the first European pediatric LT performed in Cambridge by Roy Calne in 1968[227]. Since then, pediatric LT made tremendous progress in surgical technique, medical management and related prognosis, while remaining a key clinical activity in CUSL: between 1984 and 2016 for example, 1000 pediatric LT had been performed, among which 360 living donor LT (LDLT). The 500th pediatric LDLT performed in CUSL was celebrated in January 2022.

One of the factors allowing such an important pediatric LT activity in a relatively small country such as Belgium is the historically sustained existence of "import channels" for potential recipients from other countries, notably those having limited to no access to liver transplantation on their soil. For example, Meddy Dalex described a series of 100 CUSL pediatric LT happening between 2012 and 2016 and found out that only 25 among these recipients came from Belgium, whereas 70 had been referred from non-European Union (EU) countries, mainly Algeria (23), Russia (18) and Israel (13)[228]. An interesting finding related to this varied recipient provenance is the high prevalence of pre-LT MDRB digestive carriage in this population,

which itself significantly correlates with the non-EU provenance status of the recipients: among the 100 recipients of the Dalex series, systematically screened upon first hospital admission by rectal swabbing, 44 were asymptomatic digestive carriers of ESBL- or carbapenemase-producing Enterobacterales or other MDR Gram-negative bacilli, 37 of these carriers belonging to the non-EU subpopulation (p-value: 0.012). Expectedly, early bacterial infections happening during the first 90 days after LT were frequent: 25 children suffered from at least one documented bacterial infection in this delay, for a total of 36 distinct documented infections.

The co-existence of the unresolved CIC problematic, a population in our direct clinical setting that incarnated it, and the development of our interest and technical abilities in PT led us to build and validate a study protocol called OPTICS: *Oral Phage Therapy against Intestinal Carriage of Superbugs*.

The OPTICS study was a prospective study carried between 2021 and 2023, that can be summarized in two main goals. First, a descriptive goal: similar to Dalex, to continue the description of all incoming CUSL pediatric LT recipients along demographic, clinical and microbiological criteria. Second, a feasibility study with a potential interventional component: to discover whether relevant bacterial isolates found as MDRB digestive carriages in this population could be susceptible to one or several phages that was available to us at the time, and if so, to potentially administer adapted oral PT to these patients during the pre-LT period to attempt phage-mediated targeted digestive decolonization.

The screened population consisted in all hospitalized children in CUSL for whom performing LT was either officially planned or at least considered on the short to midterm.

Among these screened patients, formal inclusion to the protocol required, by definition, the pre-LT detection of an asymptomatic digestive carriage of at least one of the following:

- ESBL- or carbapenemase-producing *E. coli* or *K. pneumoniae*

- MDR/XDR/PDR *P. aeruginosa* or *A. baumanii*

- Vancomycin-resistant *E. faecium or E. faecalis*

Accepted diagnostic method for such a digestive carriage detection included systematic screening by rectal swabbing upon first hospital admission, or incidental finding from stool culture, when performed.

The next necessary inclusion criteria then required that the carried bacterial strain (or at least one of them in the case of multi-species combined carriages) was susceptible to at least one phage that was available to us at the time, in collaboration with aforementioned Lab MCT phage collection. This susceptibility was assessed by phage spot test, a classic modality of phage susceptibility testing ("phagogram").

When both these inclusion criteria were met, inclusion was officialized if the patient and their parents (or responsible person) expressed free and informed consent, after they had been provided with age- and language-adapted information about the study protocol, and given sufficient time to ask and get responses to any questions they might have.

Even if all these inclusion criteria were met, inclusion of the patient to the protocol was irrevocably prohibited if at least one of the following exclusion criteria were present: active and documented bacterial, viral, parasitic or fungal infection at the time of considered inclusion, confirmed functional or anatomical enteropathy or colopathy (besides those directly related to the underlying chronic liver disease such as portal hypertension colopathy), confirmed or suspected allergy to any type of therapeutic bacteriophage preparation if previously exposed to, age below 3 months or above 15 years, or suspicion that application of the interventional protocol would delay LT surgery in a potentially harmful way.

The main descriptive data collected in all screened patients included clinical indication for LT, the pre-existence of a Kasai portoenterostomy, the EU or non-EU provenance of the subjects, the presence of suspected or documented post-LT infections during a 30 days delay, the identification of the one or several bacterial species detected as pre-LT asymptomatic digestive carriage, and the results of the phage susceptibility testing performed on them.

If a patient was duly included to the protocol, the interventional part of the study would then take place. Included patients would all belong to the single interventional arm of the study since this study did not achieve the clinical RCT status for aforementioned regulatory constraints (see Section II.2.2). First, the included patient would be re-tested by rectal swabbing after a 10 days delay to document persistence or spontaneous clearance of the initially detected carriage. If the carriage was still present, the protocol would continue and oral PT based on phage(s) previously selected through phage

susceptibility testing would be performed: after oral pre-medication with 500 mg (patients 3 years old or younger) or 1g (patient above 3 years old) sodium bicarbonate, 10mL of personalized phage solution (titer 10^7 PFU/mL) would be administered orally, three time a day during seven days, organized so that the last day of oral PT would fall into a 12-48h interval before LT was performed. Additional follow-up rectal swabs for carriage monitoring would be performed on the last day of oral PT and on day +10 and +30 after LT.

The existence of a pre-PT 10 days delay after which carriage persistence or spontaneous clearance would be assessed acted as a "pseudo-cross-over" precaution facing the impossibility to constitute either two proper interventional arms or a proper cross-over clinical trial. If spontaneous clearance was suspected based on a negative rectal swab after this 10-day delay, another rectal swab would be performed within three days; if again negative, the patient would be excluded from the protocol.

The primary outcome assessed in included and fully treated patients ("per protocol" analysis) was the rate of patients showing negative bacterial culture of the targeted strain(s) on the rectal swab collected on the last day of oral PT.

Secondary outcomes included :

- Rates of patients showing such negative bacterial culture on rectal swabs performed on day +10 and day +30 after LT.

- Between the last rectal swab retrieved before PT initiation and the rectal swab retrieved on the last day of PT, comparison of the mean and

variance of aggregated longitudinal ("within") evolution curves of two quantitative monitoring metrics :

- qPCR cycle threshold (Ct) of the pre-identified gene responsible for the antimicrobial resistant phenotype constitutive of the targeted digestive carriage

- 16S rRNA targeted metagenomics reads number, specific of the targeted bacterial species and estimating its quantitative abundance

Exploratory outcomes included :

- Aggregating these last items, rates of fully treated patients exhibiting a "triple negativation" profile including respectively negative post-PT bacterial culture, statistically significant lowering of qPCR Ct and statistically significant lowering of 16S rRNA reads number.

- Rates of included patients presenting suspected spontaneous clearance of a digestive carriage based on the negative bacterial culture of two distinct rectal swabs retrieved after an intervention-free 10 days delay.

The protocol is summarized in a graphical abstract in **Figure IV.2.**

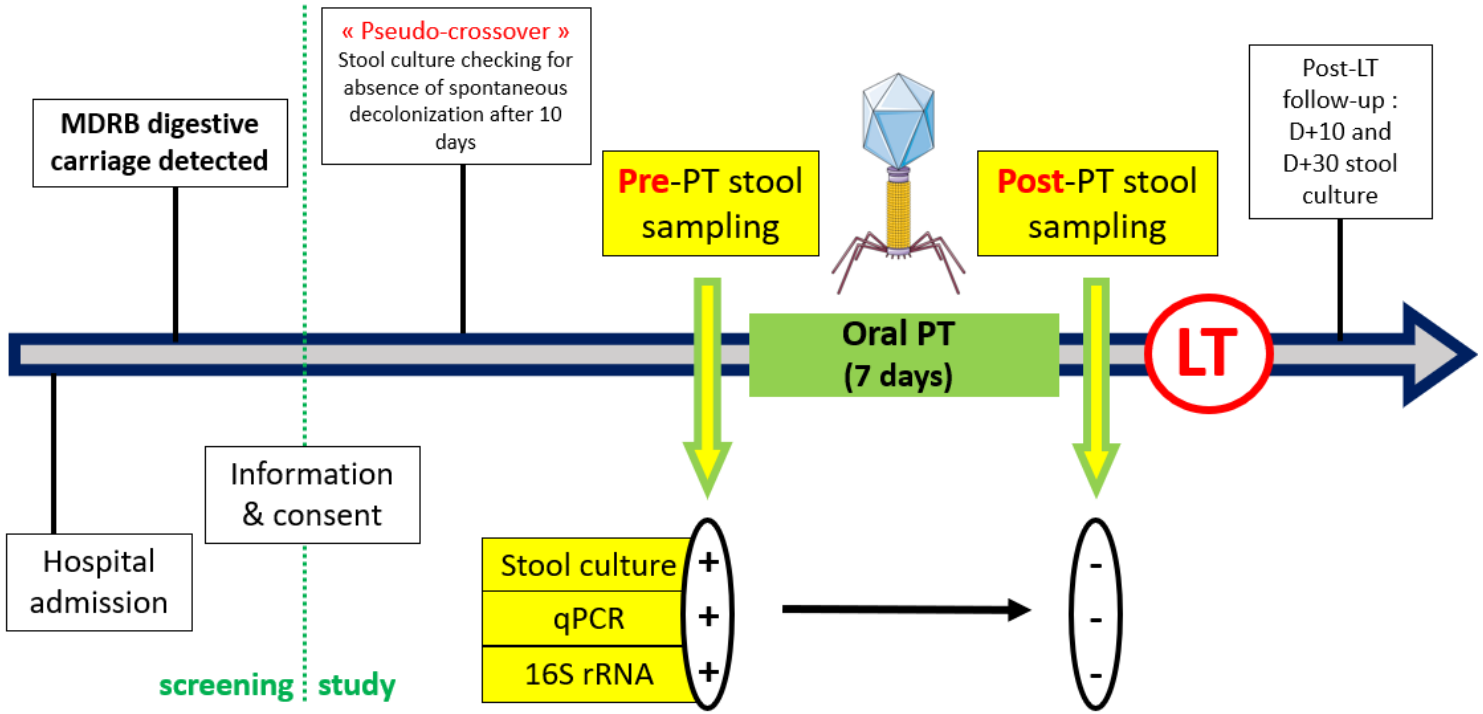


Fig. IV.2 | OPTICS study protocol graphical abstract. *MDRB, multi-drug-resistant bacteria; PT, phage therapy; LT, liver transplantation; qPCR, quantitative polymerase chain reaction; 16S rRNA designates 16S rRNA targeted metagenomics by extension*

IV.3 The OPTICS study: results

Between March 1st, 2022 and September 30th, 2023, a total of 41 potential pediatric LT recipients were screened for eligibility to OPTICS. Out of these 41 patients:

- Screening time frame has not yet been completed in 7 patients, as LT was not yet performed on them at the time of last data analysis.

- Screening time frame has been completed for the remainder 34 patients, because:

  - One was initially admitted to CUSL for pre-LT assessment, hence screened for OPTICS, but was later transferred to another hospital to perform LT.

  - One died between pre-LT assessment and the initially foreseen LT day.

  - The remainder 32 underwent a total of 33 LT procedures (one patient had to endure early re-transplantation) and completed the 30 days post-LT surveillance period.

Before detailing the aforementioned data derived from this screened population, we should first mention, however, that none of these screened patients could be included to the OPTICS study following the inclusion and exclusion criteria that we have previously detailed. The reasons for this will be detailed in the following "Discussion" section (section IV.4). Accordingly, no oral PT was administered to any of these first 41 screened patients, and

none followed the culture-qPCR-16S rRNA triple monitoring that we had foreseen, as already explained.

Nevertheless, valuable data could still be extracted from the screened population, despite the non-exploitation of the study's interventional component. Some variables will first be detailed for all 41 screened patients, then, we will discuss the more specific infection-related variables derived from the 33 LT performed among 32 patients of this screened population.

Among the 41 screened patients, first, biliary atresia still dominated as the main clinical indication for considering LT, accounting for half of the cases (20/41). Eighteen other patients harbored other LT-requiring conditions such as various types of progressive familial intrahepatic cholestasis (PFIC) (5), alpha-1 antitrypsin defect (2), maple syrup urine disease (MSUD) (2), tyrosinemia (2), Zellweger spectrum disorders (1), Alagille syndrome (1), primitive sclerosing cholangitis (1), drug-related liver damage (1), RINT1 defect-related recurrent acute liver failure (1), propionic acidemia (1), and methylmalonic acidemia (1). In the last three patients, the indication for LT appeared to be a cirrhosis of unknown origin.

Twenty-seven out of the 41 screened patients came from outside the European Union (65.8%), a rate similar to the one described in the aforementioned 100-patient CUSL pediatric LT series by Meddy Dalex between 2012 and 2016 (70%).

Twenty-two out of the 41 screened patients (53.7%) were detected as digestive carriers of at least one resistant strain of the aforementioned five bacterial species, nine out of them carrying at least two of these at the same

time. This initially provided at preliminary 33 strains of interest, among which: 17 *E. coli* (51.5%), 13 *K. pneumoniae* (39.4%), two *E. faecium* (6.1%) and one *P. aeruginosa* (3.0%)

Careful subcultures of seemingly non-pure cultures and ulterior precise species identification by MALDI-TOF (Matrix Assisted Laser Desorption Ionization - Time of Flight) revealed :

- that four *E. coli* cultures suspected to be non-pure could be each subcultured in two distinct *E. coli* colony morphologies, which were subsequently considered distinct isolates for ulterior phage susceptibility testing;

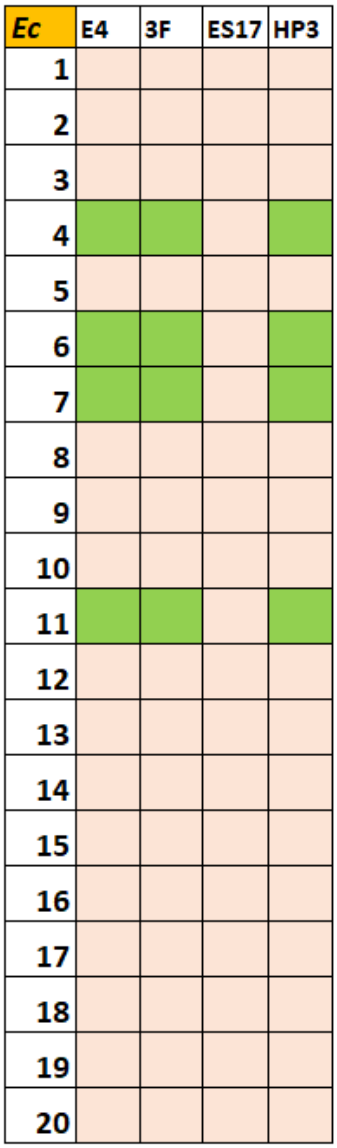
- that one of the suspected *E. coli* pure culture was an *E. coli – K. pneumoniae* co-culture;

- that another suspected *E. coli* strain actually belonged to the *P. mirabilis* species and was thus excluded from further analysis;

Eventually, a total of 20 *E. coli,* 14 *K. pneumoniae,* 2 *E. faecium* and 1 *P. aeruginosa* distinct isolates (total = 37) were screened through spot-test phage susceptibility testing. Phage susceptibility testing used a panel of available phages in collaboration with LabMCT, consisting in 4 anti-*E. coli* phages, 14 anti-*K. pneumoniae* phages, 6 anti-*E. faecium* phages and 21 anti-*P. aeruginosa* phages. Phage susceptibility was considered significant if serial dilutions showed activity equivalent to relative EOP equal or superior to 0.01 compared to the phages' respective reference bacterial host strains. Of all tested bacterial isolates, phage susceptibility to at least one tested phage was considered significant for 4/20 *E. coli* (20%), 1/14 *K. pneumoniae*

(7.1%), 0/2 *E. faecium* (0%) and 1/1 *P. aeruginosa* (100%). Full results of these phage susceptibility testing panels are displayed in **Table IV.2**.

a



b

Kp	NB2	UZG11	vB_KpnS-VAC4 (K2)	vB_KpnM-VAC13 (K2)	vB_KpnS-VAC35 (K30)	vB_KpnS-VAC51 (K64)	vB_KpnM-VAC66 (K139)	11G	15F	19/10	4A	UZG4	UZG6	UZG13
1														
2														
3														
4														
5														
6														
7														
8														
9														
10														
11														
12														
13														
14														

3342

c

Ef	KN	AQ2	AQ5	EF7	EF8	ESO25 8.1
1						
2						

d

Pa	14/1	PT07	PNM	4P	DP1	C	4K
F	PT40-1	PT40-2	3P	4A	B	J	
M	AQAPA6B	AQAPA6F	SJPA11	S10	Z10	NP3S	

Table IV.2 | OPTICS isolates phage susceptibility testing. Columns include the name of each phage tested in the respective panels and rows represent isolates of (a) *Escherichia coli*, (b) *Klebsiella pneumoniae*, (c) *Enterococcus faecium* and (d) *Pseudomonas aeruginosa*. Green squares signal significant phage susceptibility as opposed to light orange squares. *Ec, Escherichia coli; Kp, Klebsiella pneumoniae; Ef, Enterococcus faecium; Pa, Pseudomonas aeruginosa.*

As previously mentioned, 32 among the 41 screened patients benefited from LT, accounting for 33 distinct LT episodes at time of writing, one of the patients having required early re-transplantation. Ten out of these 33 LT were followed, within the first 30 days, by at least one clinically suspected episode of bacterial infection requiring antibiotic treatment. Out of these ten suspected bacterial infections, eight were considered confirmed bacterial infection after proper isolation of the causative bacterial species on a relevant microbiological sample. Three patients died within the 30 days following LT. In two of them, early post-LT infection was considered the primary factor leading to death. Five out of these eight infections involved more than one bacterial species. *K. pneumoniae* was the most frequently encountered species in these infections (5/8), followed by *E. coli* (3/8). A schematic summary of these infections is presented in **Table IV.3**.

Post-LT infections	*Ec*	*Kp*	*Ef*	*Pa*	*Ab*	*Er*	*Bv*	*Sa*	Infection type
1									Peritonitis (leading to death)
2									Sepsis (leading to death)
3									Sepsis (*Ec*), peritonitis (all)
4									Peritonitis
5									Bacteremia
6									Surgical site infection
7									Surgical site infection
8									Cholangitis

Table IV.3 | Descriptive summary of the infections happening in the 30 days post-LT in the OPTICS series. Rows designate patients and columns designate bacterial species. Boxes are colored when bacterial species is isolated in at least one relevant microbiological sample. Red color indicates lethal infections whereas orange color indicates non-lethal infections. *Ec, Escherichia coli; Kp, Klebsiella pneumoniae; Ef, Enterococcus faecium; Pa, Pseudomonas aeruginosa; Ab, Acinetobacter baumanii; Er, Enterococcus raffinosus; Bv, Bacteroides vulgatus; Sa, Staphylococcus aureus.*

Quantification of various correlations were then assessed in the OPTICS population by Fischer's exact test, which was preferred to standard chi-square test given the occasionally very limited number of events in some of the sub-categories. MDRB carriage was not significantly correlated with the post-LT development of either suspected or confirmed bacterial infections ($p = 0.246$ and 0.108 respectively).

We then postulated that this non-correlation could result from a interfering variable: surgical antibiotic prophylaxis (SAP). Indeed, pediatric LT performed in CUSL have long benefited from a specific protocol of SAP, using intravenous amoxicillin and temocillin. The use of this antibiotic combination, rather uncommon in SAP, has been empirically proposed and used by the medical and surgical teams supervising pediatric LT in CUSL for over ten years, in part due to their knowledge of a specifically high rate of ESBL-Enterobacterales among potential recipients, which temocillin could offer a reasonable empirical coverage for.

To refine our analysis, we thus retrospectively tracked back the temocillin susceptibility profile in all relevant carried isolates, routinely assessed by the clinical microbiology lab by either microdilutions or disk diffusion method, and providing a susceptible, "susceptible, increased exposure" (formerly known as "intermediate" susceptibility), or resistant result. Among the 22 carriers out of the 41 screened patients, four patients carried at least one temocillin-resistant isolate. All four of these patients completed OPTICS follow-up since three of them achieved the 30 days post-LT delay and one of them died during this delay. Additionally, one of these four patients was the one who required re-transplantation: before both transplantations in this patient, temocillin-resistant carriage was detected. In summary, five distinct LT procedures were performed in temocillin-resistant carriers. Interestingly, four of these five LT were followed by a confirmed bacterial infection in the 30 days post-LT, all of these four infections involving at least one temocillin-resistant pathogen. This five LT sub-population thus accounts for half of the post-LT early bacterial infections (4/8), whereas the 28 other LT, performed in patients carrying no detected

temocillin-resistant isolate, were followed by the remaining half of these confirmed infections. This asymmetry can be translated in three new specific Fischer's exact test associations:

- Among all 33 LT episodes, post-LT infections were significantly more likely to happen in temocillin-resistant carriers than in the rest of the patients, either non-carriers or temocillin-susceptible or temocillin-"intermediate" carriers ($p = 0.0076$)

- Conversely, excluding temocillin-resistant carriers, no significantly different association to post-LT infections was found when comparing temocillin-susceptible or –"intermediate" carriers on the one hand, to non-carriers on the other hand.

- Lastly, even when considering exclusively carriers, confirmed post-LT infection was significantly more frequent in pre-LT temocillin-resistant carriers than in temocillin-susceptible or temocillin-"intermediate" carriers ($p = 0.0307$)

It should be noted that temocillin-resistant carriage was not significantly associated with post-LT death from all causes ($p = 0.400$) nor with post-LT infection-related deaths ($p = 0.284$).

Considering associations related to geographical origin of patients, strikingly, all 22 relevant carriages were detected in patients coming from outside the EU ($p < 0.00001$). Twenty-three out of these 27 non-EU patients benefited from LT and completed OPTICS follow-up. Among the 33 LT recipients, non-EU origin was not in its own right significantly associated

with post-LT infections (p = 0.382), even though all but one of these infections happened in non-EU patients.

Lastly, among the 41 screened patients, 20 patients suffered from biliary atresia as a potential LT indication, among which 9 had previously benefited from attempted semi-conservative surgical treatment by Kasai portoenterostomy. Six of these 9 patients were MDRB carriers, which did not represent a significantly strong association between post-Kasai status and MDRB carriage either among all 41 screened patients (p = 0.4659), or only in the biliary atresia sub-population (p = 1). Post-Kasai status was not either strongly associated with post-LT infections, be it among all 33 LT patients (p = 0.1695), or among subpopulations of these 33 patients such as biliary atresia patients only (p = 0.638) or MDRB carriers only (p = 0.3563).

IV.4 Discussion

The OPTICS study was initially designed mostly as a single-arm interventional study evaluating the potential of targeted oral PT in the decolonization of pre-LT asymptomatic digestive carriage of specific MDRB. The rationale was to therefore mitigate post-LT infection risk. The study ended up failing to meet that objective, but still provided potentially thought-provoking results in the field of infectious risk management, thanks to its observational epidemiological and microbiological data.

The aforementioned interventional aspect of the OPTICS study relied on the administration of oral PT in some of the screened patients if they respected a series of inclusion and exclusion criteria: eventually, however, none of them did. Out of the 22 screened patients who were detected as potentially eligible MDRB carriers, phage susceptibility testing revealed that only six of them showed sufficient phage susceptibility to at least one of their carried MDRB to be eligible for proper inclusion to the study protocol. However, in all of these six patients, medico-surgical consensus considered that organizing pre-LT PT as described in the protocol would delay the procedure in these potentially unstable patients, representing an additional exclusion criterion. This delay for example included the delay to perform phage susceptibility testing, which was on occasion performed post-hoc, for the sake of data exhaustivity but also in anticipation of a potential need for therapeutic PT in the event of a post-LT infection.

These two exclusion criteria can and will be tackled in the future to optimize patient eligibility to this protocol, or similar future protocols. The second exclusion criterium is mostly of organizational nature, and its burden should be attenuated now that in the meantime, our team has gained experience, swiftness and flexibility in the organization and performance of strain isolation and typing, phage susceptibility testing, and potential phage products preparation and transport. The first exclusion criteria, however, appears as a tougher challenge to address. Phage susceptibility testing indeed illustrated that only six out of 37 distinct isolates showed sufficient susceptibility to at least one phage to allow for protocol inclusion. As *E. coli* and *K. pneumoniae* are by far the two leading species among these isolates (34/37), optimizing phage coverage for these two species seems like a key

challenge for the OPTICS study to be pursued in the future. The reasons for this poor phage coverage appear to differ significantly between both species. In *E. coli*, the problem might be primarily linked to the limited number of phages available in LabMCT for this species: initially, only two anti-*E. coli* phages (E4 and 3F) were available, which were later completed with phages ES17 and HP3, which we will mention again in Chapter V. This limited number of available phages is mostly attributed to the limited demand to LabMCT for anti-*E. coli* in clinical, non-research application. By experience, this is due to the fact that *E. coli* is not, at the time of writing, the most problematic pathogen in Belgium either for infections caused by specifically XDR/PDR bacteria (of which archetypal species could rather be *P. aeruginosa* or *K. pneumoniae*, for example), or for infections that are not necessarily caused by intrinsically drug-resistant bacteria but whose localization and often important biofilm formation potential lead to therapeutic dead-ends (of which the archetypal species could be *S. aureus*, for example).

However, this problematic of low demand-induced offer in phages does not hold true for the second species of interest, *K. pneumoniae*. Clinical demand for such phages is indeed higher, but building an efficient anti-*K. pneumoniae* phage collection proves extremely challenging. Indeed, the vast majority of characterized anti-*K. pneumoniae* phages suffer from high dependency to *Klebsiella* capsule type[229, 230]. *K. pneumoniae* is a gram-negative rod-shaped bacterium which is surrounded by a protective polysaccharide capsule, also called K antigen: this capsule is produced by the help of a series of enzymes, among which the glycosyltransferases, which help elongate polysaccharides by linking new oligosaccharides to them, and

whose activity is very diverse across *K. pneumoniae* strains[231, 232]. This diversity in enzymatic activity induces a diversity in capsular polysaccharide structure, these structural specificities defining a large number of so called "capsule types" or "K types". To this day, at least 138 distinct polysaccharidic combinations have been identified, among which 77 can be immunologically segregated as distinct serotypes[231, 232]. Most anti-*K. pneumoniae* phages appear to use phage-encoded depolymerases to degrade the polysaccharides of these external capsules to gain access to the surface of *K. pneumoniae*'s outer membrane for proper adsorption and initiation of the phage infective cycle (see Section II.3.2)[229]. Regrettably, these depolymerases are themselves specific to K types, conferring a usually narrow bactericidal spectrum to most anti-*K. pneumoniae* phages considered individually, the type of phage-encoded depolymerase being highly predictive of capsular tropism[229]. Even when considering this capsule-dependency limitation, choosing a phage adapted to the capsule type of a given targeted *K. pneumoniae* isolate appears as a necessary constraint, but not necessarily a sufficient one, as post-adsorptive phage resistance mechanisms also exist[229]. However, a minority of non-capsule-dependent anti-*K. pneumoniae* phages have been identified, with a broader host range, and have been found to seemingly achieve infection in a depolymerase-independent way, suggesting alternative, non-canonical infection pathways[229]. Additionally, Lourenço and colleagues have also recently illustrated that the combined use of "traditional", capsule-dependent, narrow-spectrum phages along with phages targeting non-capsulated *K. pneumoniae* isolates could result in synergistic bactericidal properties[230].

Still, capsule-dependency among anti-*K. pneumoniae* phages represents a challenge for the constitution of an efficacious phage panel, that

would ideally cover a large number of clinically relevant strains with only a handful of phages. Instead, a more realistic but far more tedious approach would be to build an anti-*K. pneumoniae* phage collection containing at least one phage for each "clinically relevant" capsule type, this clinical relevance itself being established in accordance to local epidemiology by performing, for example, large scale systematic capsule-type screening among all MDR/XDR/PDR-*K. pneumoniae* isolates encountered in Belgian tertiary care hospitals. Additionally, to mitigate the workload of phage susceptibility testing, *K. pneumoniae* isolates sent to LabMCT for phage susceptibility testing should ideally come with an already identified capsule type, so as to focus phage susceptibility testing on the sub-panel of phages known to be specific to that capsule type, and not on the whole anti-*K. pneumoniae* phage collection, which could, in time, contain more than a hundred phages.

Despite these key limitations explaining the relative failure of the interventional, phage-based aspects of the OPTICS study, other collected data still proved interesting.

Unlike a number of previously mentioned works, the two CUSL pediatric LT series, this one and the previous 2012-2016 one described by Dalex, did not illustrate significant correlation between the pre-LT digestive carriage of MDRB and the post-LT risk to develop early bacterial infection. A first limitation to this observation in the Dalex series is that they focused only on ESBL-gram negative bacilli carriage. A second limitation in this series is that the carriage-infection correlation was only established for bacterial infections of all microbiological etiologies, and not also for infections caused specifically by the previously carried MDRB. Still, as

previously mentioned, we did not either illustrate such a correlation in our smaller series. We then rapidly suspected that this could be due to a local specificity acting as an interfering variable: the systematic use of a peculiar SAP based on amoxicillin and temocillin, the latter being specifically chosen for its reliable empirical coverage of most ESBL-Enterobacterales. In the Dalex series 90 out of the 100 transplanted patients benefited from this amoxicillin-temocillin prophylaxis, while the remaining ten benefited from another SAP regimen, probably based on specific pre-LT detected carriage, though Dalex does not specifically mention it for each of these ten cases. Dalex did however not evaluate the resistance patterns of its detected MDRB carriages to these respective SAP regimens, as he did not report either on the resistance profiles of the isolated pathogens causing the post-LT infections in his series.

In our series, all patients received amoxicillin-temocillin SAP, even in the aforementioned five LT performed on patients known to be carriers of at least one temocillin-resistant strain of interest. Interestingly, four out of these five LT were followed by a documented post-LT bacterial infection (50% of all infections in our series), all of them being caused by at least one bacterial isolate exhibiting temocillin-resistance. This correlation should of course be interpreted cautiously, given the small sample size of this study and its retrospective nature, making it impossible to fully rule out the contribution of other confounding factors. Yet, this correlation might reopen the question of SAP "tailoring" in this clinical population. As described by Temkin and colleagues in their 2021 summary on the topic of SAP in patients colonized with MDR Gram-negative bacteria, literature on this topic is relatively scarce, as are official recommendations from large scale organizations[233]. Given the

relatively low incidence of post-surgical infections, building sufficiently powered RCTs to try to illustrate SAP tailoring's superiority would require thousands of patients per arm, which seems like a daunting task[233]. Yet, one example of such ambitious work is the 2019 international prospective cohort study reported by Dubinsky-Pertzov and colleagues, in which they illustrate that the use of non-tailored SAP (in their case a classic regimen including non-large spectrum cephalosporins and metronidazole) in 220 ESBL-Enterobacterales carriers led to a significantly higher rate of post-LT early bacterial infections than in the 440 non-carriers controls[234]. Such robust work retrospectively validates the intuitive use of the amoxicillin-temocillin SAP in our CUSL LT population, but it also advocates to not just stop there but pursue personalization of SAP in a carriage-specific fashion. Exposition to other, broader-spectrum antibiotics in SAP can however lead to specific toxicity, and carries a theoretical risk for antibiotic resistance selection, as previously mentioned[233]. Implementation of such tailored SAP in this CUSL population will thus be reconsidered from now on, with this risk-benefit balance in mind.

In parallel to our efforts to overcome the aforementioned challenges of pre-LT oral PT implementation, both of logistical and microbiological nature, we also wanted to investigate its potential in an experimental model. This will be discussed in the next chapter.

Chapter V: Phage-mediated digestive decolonization in a Gut-On-A-Chip model: a tale of gut-specific bacterial prosperity

Brieuc Van Nieuwenhuyse[1], Maya Merabishvili[2], Nathalie Goeders[3], Kevin Vanneste[3], Bert Bogaerts[3], Mathieu de Jode[4], Joachim Ravau[1], Jeroen Wagemans[5], Leïla Belkhir[6,7]† & Dimitri Van der Linden[1,8]†

1 - Institute of Experimental and Clinical Research, Pediatric Department (IREC/PEDI), Université catholique de Louvain - UCLouvain, Brussels, Belgium

2 - Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium

3 - Transversal Activities in Applied Genomics, Sciensano, Juliette Wytsmanstraat 14, 1050 Brussels, Belgium

4 - Bacterial Diseases, Sciensano, Brussels, Belgium

5 - Laboratory of Gene Technology, KU Leuven, Leuven, Belgium

6 - Division of Internal Medicine and Infectious Disease, Cliniques Universitaires Saint-Luc, Université catholique de Louvain - UCLouvain, Brussels, Belgium

7 - Louvain centre for Toxicology and Applied Pharmacology, Institute of Experimental and Clinical Research (IREC/LTAP), Université catholique de Louvain - UCLouvain, Brussels, Belgium

8 - Pediatric Infectious Diseases, General Pediatrics Department, Cliniques universitaires Saint-Luc, Université catholique de Louvain - UCLouvain, Brussels, Belgium

Accepted for publication at Viruses

V.1 Abstract

Preventively eradicating asymptomatic carriages of MDRB in the digestive tract has been postulated as a potential way to prevent CIC infections. However, previous attempts at such eradication using oral antibiotics or probiotics have led to discouraging results. In this work, we aimed to investigate if PT could represent a worthy alternative in this specific indication. This was studied in Gut-On-A-Chip models, complex three-dimensional cell culture models mimicking a subset of features from a simplified gut section such as tubular structure, one-way continuous luminal flow and parietal mucus secretion. These models were used to better understand bacteria-phage dynamics using two relevant bacterial species: *Pseudomonas aeruginosa* and *Escherichia coli*. Our results suggest a paradigm of multifactorial gut-specific bacterial prosperity in the pseudo-digestive environment compared to other *in vitro* conditions. Clinically relevant scenario of bacterial pre-existence in the model before phage introduction likely accentuates this phenomenon. Both bacterial species seemingly achieve phage evasion thanks to either durable genomic alterations causative of bacterial phage resistance, or to suspected dynamic gene regulation or even non-bacterial determinants. The respective contributions of these mechanisms in phage evasion might greatly vary between bacterial species. Confirming the occurrence and characterizing this dynamic gene regulation warrants further research, for example using transcriptomic or proteomic monitoring in future GOAC-mediated phage research. Lastly, in *E. coli* assays, the comparative introduction of a phage harboring unique mucus-binding properties could not shift this balance of power in various conditions,

contradicting previous findings in an *in vivo* mouse model and highlighting key differences between these models.

V.2 Introduction

In the hospital setting, specific subsets of patients may be subjected to various screening programs to detect asymptomatic carriages of certain multi- (and, by extension, extensively- and pan-) drug-resistant (MDR/XDR/PDR) bacteria, to optimize hospital-acquired infections' prevention and control. Among them, rectal swab admission screening is considered a cost-effective and efficient method to detect asymptomatic digestive carriage of certain MDR bacteria of interest, among which Gram-negative bacilli (GNB) such as extended-spectrum beta-lactamase-producing or carbapenemase-producing Enterobacterales (ESBL-PE/CPE) or other MDR-GNB such as MDR-*Pseudomonas aeruginosa*[235, 236]. These screening results then allow for preventive measures such as patient isolation, which can limit these bacteria's nosocomial spread[237].

Besides their role in limiting this nosocomial spread, rectal swab screenings' results can also benefit the screened patients themselves by refining their infectious risk profile. Indeed, the asymptomatic digestive carriage of aforementioned MDR bacteria can put the carrier at a significantly increased risk of later developing an infection caused by the carried bacteria, with the magnitude of this risk varying according to other risk factors such as intensive care unit stay, major abdominal surgery, and hematological conditions including immune suppression[218, 222, 238]. Hence, morbidity,

mortality and costs associated with these infections foster the need for preventive suppression strategies of these carriages. However, previous suppression strategies such as oral antibiotics decolonization, oral probiotics regimens as well as limited experience of FMT have yielded disappointing results, reaching temporary suppression at best rather than durable eradication[222]. In addition, oral large-spectrum antibiotics regimens also present potentially serious side effects regarding gut microbiota modification and AMR induction, and FMT, though generally safe, has on rare occasions led to serious and sometimes fatal adverse events[222, 239]. This discouraging issue can thus be considered as an unmet medical need, for which alternative or complementary suppression approaches warrant further research. PT has been mentioned among them[222].

Both case-based and trial-based literature unanimously report the general safety of PT, regardless of administration route, though reversible and non-clinically-significant cases of transaminitis have been reported on rare occasion[126, 189, 240]. This favorable safety profile might be partly attributed to phages' very narrow bactericidal spectrum, conditioned by complex bacterial surface receptor recognition. This makes phages ideal candidates for species-specific bactericidal therapy, minimizing collateral damage on commensal microbiota.

PT can be delivered in a number of administration routes to target various body compartments, such as topical administration on surface wounds, *in situ* instillations in abscess cavities, pulmonary nebulization, or intravenous infusion. However, among them, oral administration of phages to target bacteria in the digestive tract is notoriously challenging, and has

occasionally yielded disappointing results in both animal and human applications[124, 241]. Interestingly, this contrasts with the well-documented massive presence and impact of the resident gut commensal phage community, whose complex role in shaping the bacterial compartment of the gut microbiota in both health and disease is increasingly investigated[242, 243, 244]. Green and colleagues recently illustrated that the digestive tract's inhospitable nature towards oral PT was at least partly caused by one key inhibitory compound: mucin[241]. Moreover, they showed that this inhibitory effect could be surpassed by the administration of a phage featuring unique mucus-binding properties (MBPs), resulting in significantly increased lytic power against a gut-colonizing *Escherichia coli* strain (subtype ST131) in both *in vitro* and mice models, compared to another anti-*E. coli* phage devoid of MBPs.

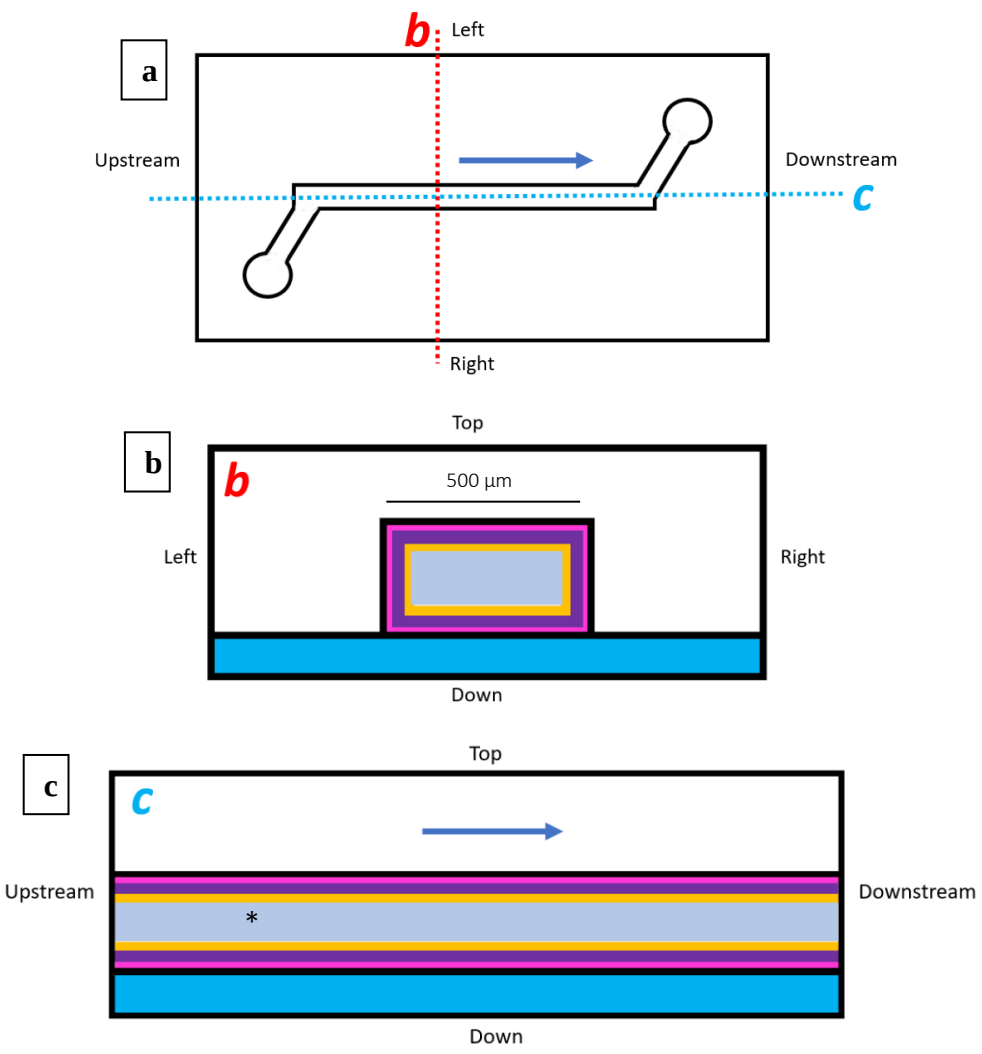
In this paper, we translate the clinical challenge of phage-mediated targeted digestive decolonization into a complex *in vitro* pre-clinical model: the Gut-On-A-Chip (GOAC). GOACs are part of a larger family of microfluidic three-dimensional tubular cell culture models: human Organs-On-Chips (OOCs), whose development has been directed and published by Donald Ingber and colleagues since 2010[245]. Since then, they have published thorough microfabrication and handling protocols that allowed others to reproduce these models and adapt them to their specific research needs and organs of interest[246]. Some have since brought proof-of-concept that GOACs can be fine-tuned to high degrees of complexity, aiming for maximum physiological relevance, including the ability to sustain growth of a complex pseudo-gut microbiota[247]. Others, including phage researchers, have used simpler GOAC designs to model a smaller subset of chosen key

characteristics while not relying on complex materials and scarce technology[248, 249]. Here, taking inspiration from the latter, we use custom GOACs to investigate quantitative and qualitative aspects of phage-bacteria dynamics in gut-like conditions, including the impact of phage MBPs, focusing on two bacterial species of interest: *P. aeruginosa* and *E. coli*.

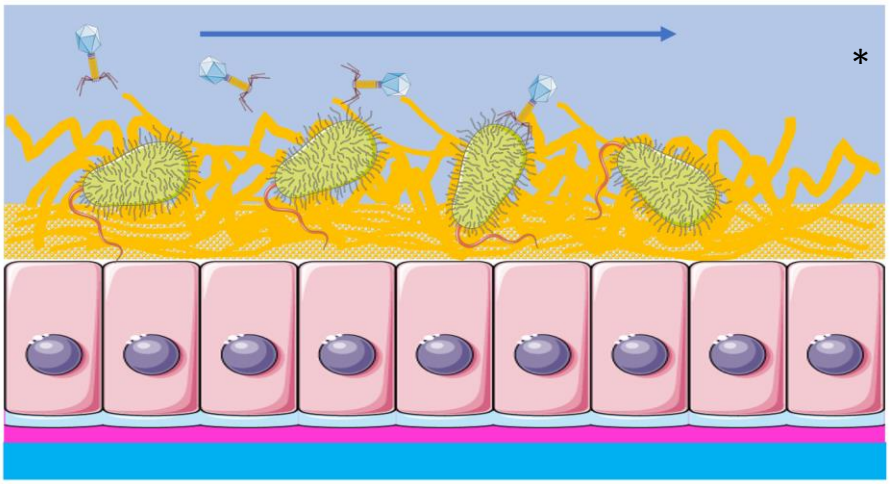
V.3 Materials and Methods

V.3.1 GOAC microfabrication – inorganic scaffold assembly

Our GOAC microfabrication protocol results from a mix of previously described elements from the aforementioned works of Huh et al., 2013, Jalili-Firoozinezhad et al., 2019 and Chin and Barr, 2019 as well as occasional personal adaptation through trial and error. We chose to work with single-channel GOACs similar to the one described in Chin and Barr, 2019, as opposed to the double-channel GOACs described by Huh et al., 2013 and Jalili-Firoozinezhad et al., 2019. A schematic view of GOACs used in our work is displayed in **Figure V.1.**



d



e

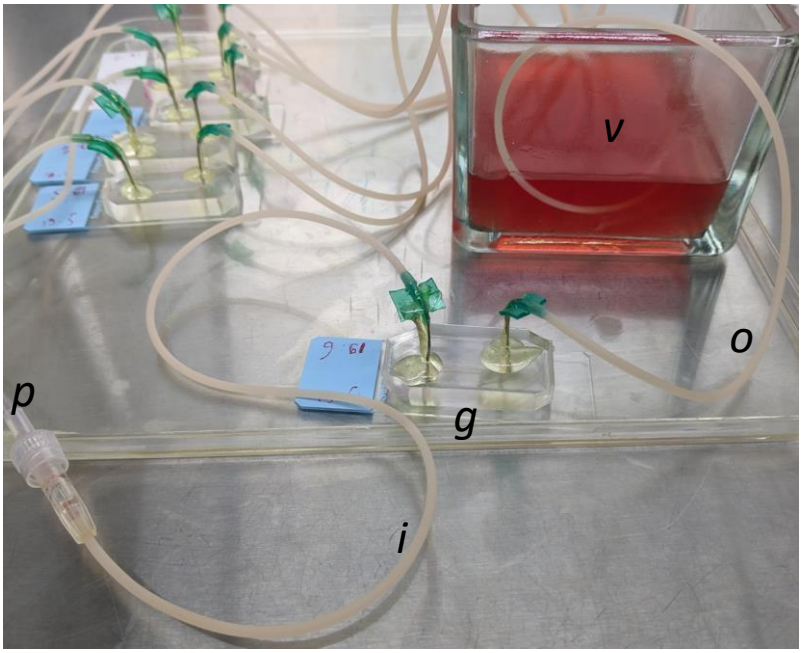


Fig. V.1 | Schematic and photographic views of the Gut-On-A-Chip (GOAC). (a) Schematic upper view of the GOAC pattern. Lower left and upper right circles represent inlet and outlet pores respectively, between which the GOAC channel is carved. Dark blue arrow represents the direction of the microfluidic flow of culture medium in the GOAC. Dotted line axes b (red) and c (blue) are used for further schematic representations. **(b)** Transversal section in the GOAC along axis b. The floor of the GOAC is a glass slide (blue) on which a carved polydimethylsiloxane (PDMS) chip (white) is sealed, between which is delimitated the GOAC channel itself. The several concentric layers of the GOAC's "gut wall" are represented, from outer to inner: extra-cellular matrix (pink), epithelium (violet), parietal mucus layer (yellow), central flow of culture medium (grey). **(c)** Longitudinal section in the GOAC along axis c. The elements are similar to those of the transversal section. **(d)** Schematic zoom in a segment (*) of the longitudinal section. In this work, the GOAC model is designed to recapitulate the scenario of PT targeting bacteria in the digestive micro-environment, which includes a parietal mucus layer. **(e)** Picture of a GOAC. The GOAC itself (g) is connected to inlet (i) and outlet (o) winged catheters, respectively introducing and evacuating all reagents to and from the GOAC. Winged catheters' needles are manually curved to be punched in inlet and outlet pores forming a 90° angle with the PDMS chip surface. At the point where the needles are punched into the PDMS chip, epoxy glue is applied to ensure impermeability. Culture medium is infused by a microfluidic pump connected to the inlet catheter by a connecting catheter (p). Egressing medium coming out of the GOAC through the outlet catheter is evacuated in a glass collecting vessel (v) filled with a base level of 70% v/v isopropyl alcohol (IPA).

The general scaffold of the single-channel GOAC relies on the bonding of a polydimethylsiloxane (PDMS) chip onto a glass slide, between which is delimitated the GOAC's internal channel volume, carved into the PDMS chip. This requires the creation of a custom mold in which PDMS will be poured and shaped to the desired pattern. Our mold was built using a plastic 3D printer (UltiMaker 3, UltiMaker B.V., Netherlands) and associated software (UltiMaker Cura, v4.11, UltiMaker B.V., Netherlands) after modeling the desired pattern file through an online 3D-printing-modelization software (Tinkercad, Autodesk Inc., CA, USA). PDMS and its curing agent (Sylgard-184 Silicone Elastomer Kit, Dow Inc., USA) are then poured in a disposable plastic weighing dish at a recommended 10:1 mass ratio and thoroughly mixed by hand using a stainless steel lab spatula during 5 minutes. The mixture is then let to rest at room temperature for about 30 minutes before residual bubbles are removed with the tip of a sterile needle.

The PDMS mixture is then manually poured in the mold at the most gentle and constant pace possible until the mold is full. The full mold is then let to rest and cure at room temperature for at least 36 hours before the fully solidified PDMS chip is carved out of the mold, with the help of a surgical blade or a lab spatula. Between each use, the mold is thoroughly washed by being brushed under tap water followed by 70% v/v isopropyl alcohol (IPA) (20842.330, VWR international, PA, USA) to ensure full clearance of PDMS residue, then let to evaporate under a fume hood until completely dry before the next use. Carved PDMS chips are then further cured in a dry incubator at 60°C for at least one hour, with the channel side up. Their four corners are trimmed using a surgical blade to give the chips an octagonal shape, while ensuring cuts do not get close from the molded channel pattern. Finally, chips

are bathed in 70% v/v IPA and let to dry fully before storage in any clean container.

Plasma bonding of the PDMS chips to glass slides (Superfrost Plus, Thermo Scientific Menzel-Gläser, Germany) is then performed. Right before plasma treatment of the PDMS chip and the glass slide, the glass slide is gently wiped clean with 70% v/v IPA and let to dry fully while visually ensuring the PDMS chip and wiped slide are devoid of any residue or dust particle. The PDMS chip (channel side up) and the glass slide are placed in the barrel of the plasma cleaner (TePla 300E, PVA TePla, Germany). The venting valve of the plasma cleaner is opened on high throughput so as to minimize post-plasma venting delay. O_2 plasma treatment is initiated at 70 watts for 45 seconds. As soon as venting is complete and the plasma cleaner's door can be opened, the glass slide is swiftly taken out while leaving untouched the plasma-treated area intended to bond to the PDMS chip. The PDMS chip is then also taken out without touching its plasma-treated face (channel face), then the respective plasma-treated faces of the PDMS chip and the glass slide are manually pressed against each other. The plasma-induced surface activation effects wear off quickly: the delay between door opening and face-to-face application should not exceed ten seconds. Both parts are pressed firmly together using fingers without moving for at least 30 seconds. Then, to strengthen the plasma bonding, annealing of the assembled chip is performed by placing it in an oven at 80°C for at least two hours. The chip is then taken out of the oven and stored in a clean container for later use. These chips form the inorganic scaffold of the future GOACs.

V.3.2 GOAC microfabrication – cell culture

This part of the GOAC microfabrication can be divided into three successive steps: i) inlet and outlet catheters connection, ii) extra-cellular matrix (ECM) coating and iii) cell seeding.

Inlet and outlet catheters are needed to respectively introduce and evacuate all reagents, cells, bacteria and phages from the GOAC, including the continuous unidirectional flow of culture medium infused in the GOAC to sustain cell growth (see below): they form an upstream and downstream tubular continuity with the actual GOAC channel inside the chip. Winged 21G catheters (21BLK03, Terumo, Japan) are used after cutting their wings short with a surgical blade and manually bending their needle to give it a smooth ~90° curvature. The curved needles are then manually punched inside the PDMS chip's inlet and outlet pores, forming a ~90° angle with the chip's upper surface. Punching needles sufficiently deep and accurately inside the channel pores is critical for future top-to-bottom permeability of the system. To ensure needle-PDMS junction is durably impermeable, once the needles are punched in place, needle-PDMS junctions at the surface of the chip are covered in two-components epoxy glue (Super Mix Universal, Power Epoxy, Pattex, Henkel, Germany) which is then let to solidify at room temperature for at least one hour. Chips are then placed under UV light overnight for sterilization.

The GOAC's inner walls are then coated with ECM solution. The end of the GOAC's outlet catheter is placed in a collecting vessel that will collect egressing fluid from the GOAC from then on. When working on a series of several GOACs at the same time, GOACs seeded with different cells, bacteria

or phages should always have their outlet catheters placed in separate collecting vessels, to mitigate cross-contamination risk. A base level of 70% v/v IPA is poured in this collecting vessel for further instant sterilization of the egressing fluid. To prepare 10mL of ECM solution under the laminar flow, the following refrigerated (4°C) reagents are poured in a 50mL sterile tube (Cellstar, Greiner bio-one, Germany) put in ice: 9800 µL of serum-free DMEM (41965- 039, Gibco, Thermo Fischer Scientific, USA), 180 µL of rat tail type I collagen (354236, Corning, USA) and 20µL of Matrigel (354234, Corning, USA). This ECM solution is then gently mixed by manually shaking the closed tube before putting it back in ice. This refrigerated ECM solution must be used immediately. A 3 mL sterile plastic syringe is loaded with 1,5 mL of ECM solution. Loading the syringe very slowly reduces bubble formation. The filled syringe is then connected to the GOAC's inlet catheter before the ECM solution is gently infused through the GOAC, until egressing ECM is visible at the end of the outlet catheter. A visual check ensures no visible bubble is present in the ECM-filled GOAC, since these spots would then remain uncoated. The ECM-filled GOAC is placed in a humidified cell culture incubator (37°C ; 5% CO_2) overnight with the ECM syringe still connected to the inlet catheter.

The next day, the ECM excess fluid is gently flushed out of the GOAC by slowly instilling, with a syringe connected to the inlet catheter, 1,5mL of the following culture medium that will be used from then on ("FULL medium"): DMEM supplemented with 20% fetal bovine serum (10270-106, Gibco, Thermo Fischer Scientific, USA), 1% penicillin-streptomycin (15140-122, Gibco, Thermo Fischer Scientific, USA) and 1% non-essential amino acids (11140-050, Gibco, Thermo Fischer Scientific, USA). The GOAC is

then ready for cell seeding. In this work, according to tested conditions (see Results section), GOACs were either seeded with 100% Caco-2 cells (HTB-37, American Type Culture Collection - ATCC, USA) or with 50%-50% Caco-2/LS 174T cells (CL-188, ATCC, USA). Once, a triplicate of GOACs was seeded with 100% LS 174T cells for the purpose of comparative ELISA validation only (see below), but were not used for infection assays. Caco-2 cells and LS 174T cells required for GOAC seeding are retrieved by trypsinization, centrifugation and resuspension of 90% confluent T175 culture flasks previously cultured with FULL medium. With the help of trypan blue cell counting calibration, a 1mL sterile syringe is loaded with 1mL of cell suspension at a total cell seeding density of about $1,5x10^7$ cells/mL. This syringe is then connected to the inlet catheter and instilled in full in the GOAC ; to ensure cell delivery in the GOAC is sufficient, this was performed with the GOAC's channel under a microscope. The GOAC is then put back statically in the incubator to ensure cell adhesion to the GOAC's walls, with the 1mL syringe still connected, during four hours. The GOAC is then instilled with 1 mL of FULL medium, to flush out unattached cells from its lumen. The GOAC is then put back statically in the incubator overnight, with the flushing syringe still attached, to ensure cell growth to epithelial confluence.

The next day, the GOAC is ready to be put under microfluidic infusion. Microfluidic infusion is ensured by a 12-channel microfluidic pump (Braintree Scientific, USA) in which 3 mL sterile syringes containing FULL medium are loaded. The GOAC is taken out of the incubator and its inlet catheter is connected to the 3mL syringe loaded in the pump with the help of a long connecting catheter (PN3120, CAIR L.G.L., France) also pre-filled

with FULL medium. The connecting catheter's length ensures the GOAC can still be moved in and out of the incubator while the pump stays outside the incubator. The GOAC is placed back in the incubator, with the connecting catheter passing through the incubator door's rubber joint. The pump is started with an infusion rate set to 60 µL/h (1 µL/min) for 72h, to sustain epithelial growth and parietal mucus secretion under microfluidic conditions. At least 12 hours before inoculating the GOAC (see next section), the medium has to be switched from FULL to antibiotic-free medium ("NAB medium"), which has the same composition as FULL medium except for the absence of 1% penicillin-streptomycin. To connect the GOAC to NAB medium for these more than 12 hours, the connecting catheter is first disconnected from the GOAC's inlet catheter and flushed of its inner FULL medium with new NAB medium using a 3mL syringe loaded with NAB medium. This 3 mL syringe is then fully refilled with NAB medium, connected again to the connecting catheter, and loaded in the pump. The downstream end of the connecting catheter can then be reconnected to the GOAC's inlet catheter. This ensures NAB medium is delivered right from the inlet catheter once the pump is re-started. During these more than 12 hours of NAB medium infusion, the pump rate is doubled to 120 µL/h (2 µL/min).

V.3.3 GOAC microfabrication – bacteria and phage introduction and monitoring

After at least 12 hours of NAB medium infusion, bacteria and phages can be introduced in the GOAC. In this work, GOACs were either inoculated

with *P. aeruginosa* strain CN573 and corresponding anti-*P. aeruginosa* phage named PNM (both sourced from Laboratory of Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium), or with *E. coli* subtype ST131 and corresponding anti-*E. coli* phages named HP3 and ES17 (all three sourced from Baylor College of Medicine, Texas, USA).

According to the desired test condition (see Results section), GOACs are either infected with $1x10^7$ CFU (colony forming unit)/mL or $1x10^5$ CFU/mL bacterial solutions. Prior to introduction in the GOAC, both *P. aeruginosa* and *E. coli* are subcultured in a 37°C incubator overnight on self-made LB agar slants. The slant is then washed by adding 5 mL of NAB medium and vortexing the slant tube at high speed for 10 seconds, providing an initial bacterial solution that is then systematically titrated by serial dilutions in phosphate-buffered saline (Dulbecco's PBS, PBS-1A, Capricorn Scientific, Germany) and plating on 5% sheep blood Columbia agar plates (254071, Becton Dickinson, USA). The agar plates are incubated overnight and the titer of the original bacterial suspension is determined *post-hoc* by CFU counting. By experience, this initial solution's titer is reliably comprised in the $1\text{-}2x10^9$ CFU/mL interval for both bacterial species, and thus diluted 100 and 10000 times to reach aforementioned desired titers of $\sim1x10^7$ and $\sim1x10^5$ CFU/mL, respectively. Variation among the exact infective titers (determined *post-hoc* the next day by plate CFU count, as explained) is neutralized by standardizing each initial titer on a conventional zero and expressing subsequent variation of the GOAC's infection on a log10 basis (see next section - Statistics).

Phages are similarly titrated to establish their PFU/mL titer using standard double agar overlay (DAO) method[250]. Knowing the respective CFU/mL and PFU/mL titers of the instilled bacterial and phages solutions, multiplicity of infection (MOI, the ratio of phages per bacteria in presence) can be determined when applicable: in this work, tested MOI is either 1 or 10. In preventive assays in which bacteria-free GOACs are pre-treated with phages, the inner GOAC volume is filled with phage solution at 10^8 PFU/mL then incubated statically at 37°C/5% CO_2 before being reconnected to the pump and washed with NAB medium either perfused at 1mL/h during 90 minutes ("hard wash" protocol), or at 120µL/h during 12h ("soft wash" protocol).

According to tested conditions, aforementioned bacterial loads and phage loads are introduced in the GOAC either by direct manual syringe instillation before immediately reconnecting the GOAC to the pumping system, or by pump-instillation starting from the pump's syringe all the way through the connecting catheter and the GOAC. "Day 0" always marks the time of initial bacterial introduction to the GOAC. Evolution of the model's bacterial titer is then followed every 24h for 72h. To achieve this monitoring, 10µL of egressing fluid are pipetted out of the outlet catheter terminal cupule by inserting a fine pipette tip inside lumen of the end of the outlet catheter. By serial dilutions of this 10µL output, daily in-model CFU/mL counts are established using the same spread plate and colony count technique as mentioned above.

V.3.4 GOAC - Statistics

GOAC statistics are computed using SPSS (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp). Between and within comparisons of the GOACs' bacterial counts expressed in log10 basis over the days are assessed through repeated measures ANOVA (ANalysis Of VAriance) tests, the "within" variable being time (days) and the "between" variable being the different tested conditions. Greenhouse-Geisser correction is automatically applied if Mauchly's sphericity test's null hypothesis is not met. ANOVA main effect's comparison is obtained after Bonferroni correction. Post-hoc multiple paired comparisons are generated after Bonferroni correction. All error bars on graphs represent 95% confidence intervals (CI95). Significance is always based on $\alpha = 0.05$. All tested conditions are biologically-independent triplicates (i.e. independent GOACs ; n=3, generally tested in at least two different runs to mitigate batch effect), each of these conditions' CFU/mL output being itself subjected to technological duplicate (mean count based on spread plating on two agar plates). These evolutions of log10 basis counts are centered on the aforementioned control count of introduced CFU/mL at day 0, which is subtracted to express all further values in comparison to a conventional zero: graphs thus represent the loss, gain or *status quo* of bacterial titer in the model compared to the bacterial titer initially instilled in the model.

V.3.5 GOAC - Visuals

To illustrate the tissue architecture of the GOACs, direct in-GOAC coloration were performed using Alcian Blue, staining acidic mucins. We performed a

classic Alcian Blue staining protocol usually performed on histological sections, but by infusing and flushing the reagents directly in the GOAC channel instead, via the aforementioned inlet catheter. We used Alcian Blue 8GX (361180-0005, RAL Diagnostics, France). The GOAC on which staining is performed is first gently flushed manually with phosphate-buffered saline (Dulbecco's PBS, Capricorn Scientific GmbH, Germany), then infused with 4% v/v paraformaldehyde (Q Path, VWR chemicals, VWR international, PA, USA) and let to fixate statically for 10 minutes at room temperature. GOAC is then flushed with PBS again before mordanting is performed infusing 3% v/v acetic acid (based on Acetic Anhydride, ref 33214-M, Sigma-Aldrich, Germany) before 3 min of static incubation at room temperature. Alcian Blue (1% v/v) is then infused in the GOAC and let to incubate in a dry incubator at 37°C for 30 minutes, before being finally flushed manually with distilled water. Stained GOACs were then directly observed through light microscopy. Their stained inlet catheters, found to often contain similarly cellularized segments, were used to obtain 3µm-thick histological sections after a classic tissue preparation and paraffin embedding protocol.

V.3.6 OmniLog assays - bacterial growth kinetics

To compare GOACs bacterial dynamics to other *in vitro* conditions over an identical timeframe of 72h, bacterial growth curves were established using an OmniLog incubator system (Biolog, Hayward, CA, USA). Experiments were done in 96-well plates in a final volume of 200 µl of LB medium supplemented with 100 times diluted tetrazolium dye mix A, according to the manufacturer’s instructions. Control assays were also

performed using 20%-FBS-supplemented LB instead, mixed with the same dye. Bacteria were added at a concentration of 10^5 CFU/well, calculated based on OD (600 nm) measurements (with an OD of 0.5 corresponding to 4×10^8 CFU/ml, on average), which were validated using a classical plate culture method. MOI tested in OmniLog assays was 1 for *P. aeruginosa* assays and either 1 or 10 for *E. coli* assays.

OmniLog data were analyzed using OmniLog data Analysis Software (v1.7). OmniLog growth curves based on these results represent bacterial proliferation in various conditions, presented through relative units of cellular respiration, over time. Error bars in these graphs represent +/- 1 standard deviation of the mean. Positive and negative control conditions were replicated in 16 wells in each assay. In LB-only assays, *P. aeruginosa* with PNM at MOI = 1 was replicated in 32 wells. *E. coli* assays with either phage ES17 or HP3 were replicated in 24 wells at MOI = 1 and in 8 wells at MOI = 10. In control assays using serum-supplemented-LB, all bacteria-MOI combinations were replicated in 24 wells. Comparison of bacterial "escape" growth rate between OmniLog assays and GOAC assays is performed by Fischer's exact test.

V.3.7 Bacteria and phages: description and variant typing

Two bacterial species were used in this work: *P. aeruginosa* (strain CN573) and *E. coli* (Extraintestinal Pathogenic *E. coli* – ExPEC - subtype ST131 isolate JJ1901). *P. aeruginosa* was subjected to only one phage in this work, lytic phage PNM of the Autographiviridae family (GenBank :

OP292288): both *P. aeruginosa* CN573 and phage PNM samples were obtained from the Laboratory of Molecular and Cellular Technology (LabMCT, Queen Astrid Military Hospital, Brussels, Belgium). *E. coli* was subjected to two lytic phages: phage ES17 and phage HP3. Phage HP3 (GenBank : GCA_002619885.1) is a lytic *Escherichia*-phage member of the Straboviridae family, possessing a myovirus morphology and depending at least partly on *E. coli* LPS as a bacterial surface receptor[251]. The more recently described Phage ES17 (GenBank : GCA_009744855.2), whose bacterial receptor is not yet characterized, is another lytic *Escherichia*-phage member of the Gordonclarkvirinae subfamily and Kuravirus genus, and displays an atypical podovirus-like morphology featuring an elongated capsid and short tail fibers[241, 252]. In addition, phage ES17 has been recently described for its MBPs compared to their absence in phage HP3[241]. Both of these phages along with the *E. coli* ST131 JJ1901 strain were obtained from the Baylor College of Medicine (Houston, TX, USA).

Some experiments in this work have generated variants of the aforementioned bacterial strains, initially identified on a visual basis by examining colonies on aforementioned 5% sheep blood Columbia agar plates. Some of these variants have been screened for acquired phage-resistance by re-performing aforementioned DAO assays to establish relative EOP, the relative ability of a given phage load to achieve lysis plaques on a bacterial lawn of a given bacterial isolate compared to the phage's reference bacterial host, expressed as a ratio theoretically comprised between 0 and 1. All of the analyzed variants were confirmed to belong to their alleged bacterial species by MALDI-TOF. Some variants of *P. aeruginosa* and *E. coli* were also sequenced to try to investigate potential genomic basis for their phenotypic

modifications: for reasons of chronological and technical constraints as well as respective expertise, *P. aeruginosa* and *E. coli* sequencing were performed by different teams, using different protocols. *P. aeruginosa* sequencing was performed as follows: total genomic DNA was extracted using the MagCore Genomic DNA Bacterial Kit (RBC Bioscience, Taiwan). The Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA) was used to prepare isolate sequencing libraries. Subsequent short-read sequencing was performed using Illumina MiSeq sequencing (Illumina, San Diego, CA, USA) according to the manufacturer's instructions to produce 2 × 250 bp paired end reads on DNA prepared with the MiSeq Reagent Kit v3 (Illumina). In parallel, long-read sequencing was performed using an Oxford Nanopore Technology MinION equipped with an R9.4.1 flow cell and the SQK-RBK004 rapid barcoding kit. Guppy (v6.4.6) was then used with the dna_r9.4.1_450bps_sup.cfg configuration file for basecalling in super high accuracy mode and demultiplexing.

Trimmomatic (v0.38) was used to trim the Illumina MiSeq reads with the following options: "LEADING" set to 10, "TRAILING" set to 10, 'SLIDINGWINDOW' set to "4:20," "MINLEN" set to 40, and "ILLUMINACLIP" set to "NexteraPE-PE.fa:2:30:10"[157]. The quality of the trimmed reads was checked using FastQC (v0.11.7)[253]. Hybrid assemblies were generated following the recommendations of Wick et. al.[254] for automating the generation of long-read first hybrid assemblies. Briefly, long-reads were trimmed using Filtlong (v0.2.0) (https://github.com/rrwick/Filtlong) and used to generate de novo assemblies using Flye with the '--genome-size' parameter set to 6,230,593 and the other parameters left at their default values (v2.9.1)[255]. The resulting assemblies

were polished using Medaka (v1.7.3) (https://github.com/nanoporetech/medaka) with the 'r941_min_sup_g507' model, followed by Polypolish (v0.5.0)[256] and POLCA (v4.1.0)[257]. BWA (v0.7.17) (Li and Durbin, 2009) was used to map the reads to the draft assemblies using the default options, with forward and reverse reads mapped separately.

SNPs and indels were detected using Snippy (v4.6.0) (https://github.com/tseemann/snippy) and Samtools (v1.13)[258] with the Bakta (v1.8.2)[259] annotated reference genome hybrid assembly and the trimmed short-reads as inputs. Detected variants were confirmed by manual investigation in IGV[260]. Large structural deletions were detected by generating coverage plots of the short-reads using tinycov (v0.4.0) (https://github.com/cmdoret/tinycov) on the BAM files generated by Snippy. Mummerplots (v3.5)[261] was used to confirm the large chromosomal deletions and to search for other large insertions and inversions by comparing the variant hybrid assemblies to the reference hybrid assembly (i.e., the genome of the original strain before selection). Pan-genome analysis was performed on the hybrid assemblies using Prokka (v1.14.6)[262] and Roary (v3.13.0)[263] with the '--mafft' option enabled, the minimum identity set to 95%, and paralogs not split ('-s'). Data manipulation and visualization for the circular chromosomic view used R (v4.2.2) and the tidyverse (v1.3.2)[264] and circlize (v0.4.15)[265] packages.

E. coli sequencing was performed as follows: total genomic DNA was extracted from the *E. coli* isolates using the DNeasy UltraClean Microbial kit (Qiagen). The DNA was prepared for long-read sequencing (Rapid barcoding

kit SQK-RBK114.24, Oxford Nanopore Technology) on a MinION equipped with an R10.4.1 flowcell. Basecalling of the nanopore data was carried out using Guppy (v6.3.8)[266] in super high-accuracy mode, followed by demultiplexing also with Guppy. The reference genome was assembled using Unicycler[159] (v0.5.0)[267] and annotated using Prokka[262] (v1.14.6). SNP calling was done with snippy (https://github.com/tseemann/snippy) (v4.6.0) using the reads of the variants against the annotated reference genome. Coverage was visually inspected using UGene (v44.0)[268] after mapping the reads on the reference with Bowtie2 (v2.5.0)[269].

V.3.8 ELISA mucin quantification assays

Quantitative ELISA tests were performed on GOAC triplicates to assess their mucin secretion profile based on the content of the egressing culture medium coming out of their outlet catheter. Three triplicates of three different cell compositions were analyzed: 100% Caco-2, 50%/50% Caco-2 – LS 174T, and 100% LS 174T. Standards, controls and samples were completed in technical duplicates. All GOACs were analyzed 120 hours after cell seeding: after this delay, GOACs egressing fluid was collected at a pumping speed of 1.0mL/h for 30 minutes (volume of 0.5mL required for the assay) and centrifuged at 2,000 x g for 10 minutes to remove debris, then stored at -20°C. Both protocols were then carried out according to the manufacturer's instructions considering the following points: For the Human MUC2 SimpleStep ELISA® Kit (ABCAM ab282871), the samples were diluted 1:2 into Sample Diluent solution. For the Human Mucin 5AC SimpleStep ELISA® Kit (ABCAM ab303761), the samples were diluted 1:20

into Sample Diluent solution. The optical density measurements were recorded at 450nm during 1 second by well. The analysis was performed by subtracting the average blank control standard absorbance value from all other absorbance values. Four-parameter logistic (4PL) curve fit was created by plotting the absorbance values for each standard concentration plotted on log-log axes. The concentration of the target protein in the sample was then determined considering the sample dilution factor. Mean comparison between tested groups was obtained by univariate ANOVA using SPSS. Post-hoc multiple paired comparisons are generated after Bonferroni correction.

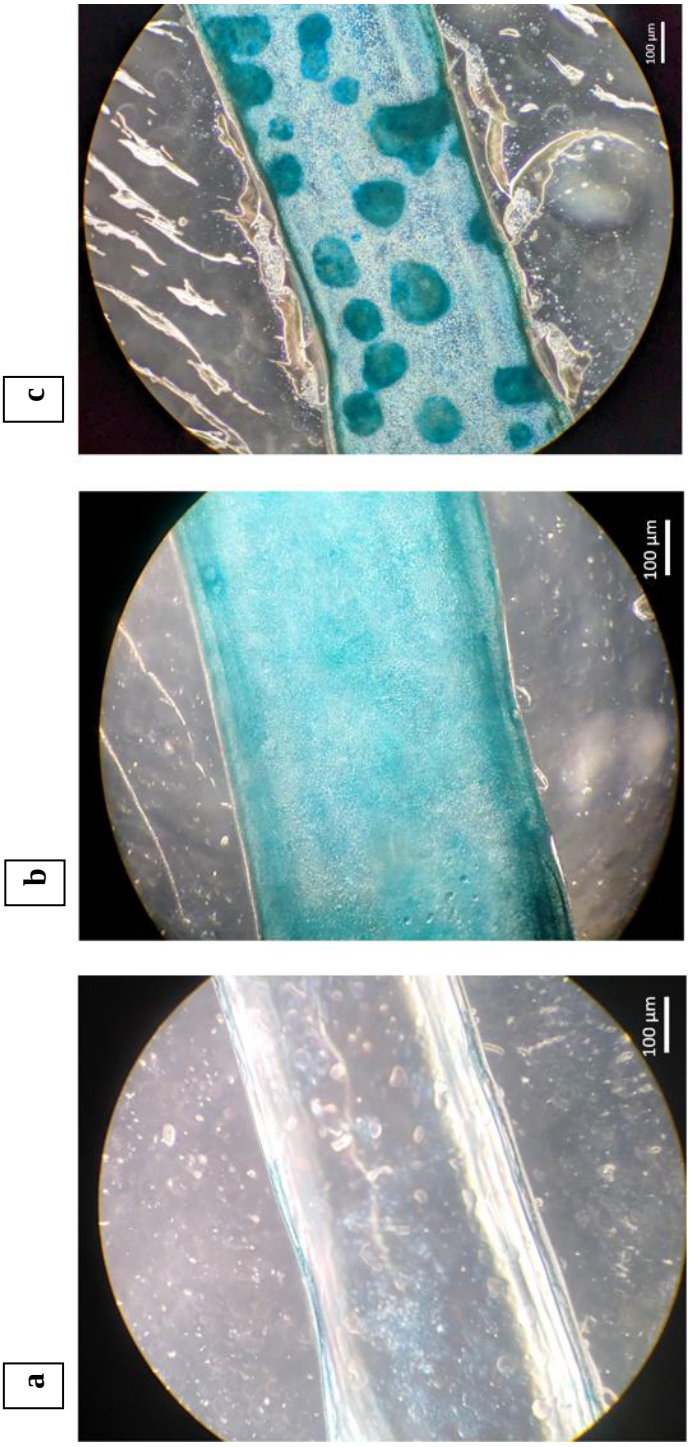
V.4 Results

V.4.1 GOAC cell culture

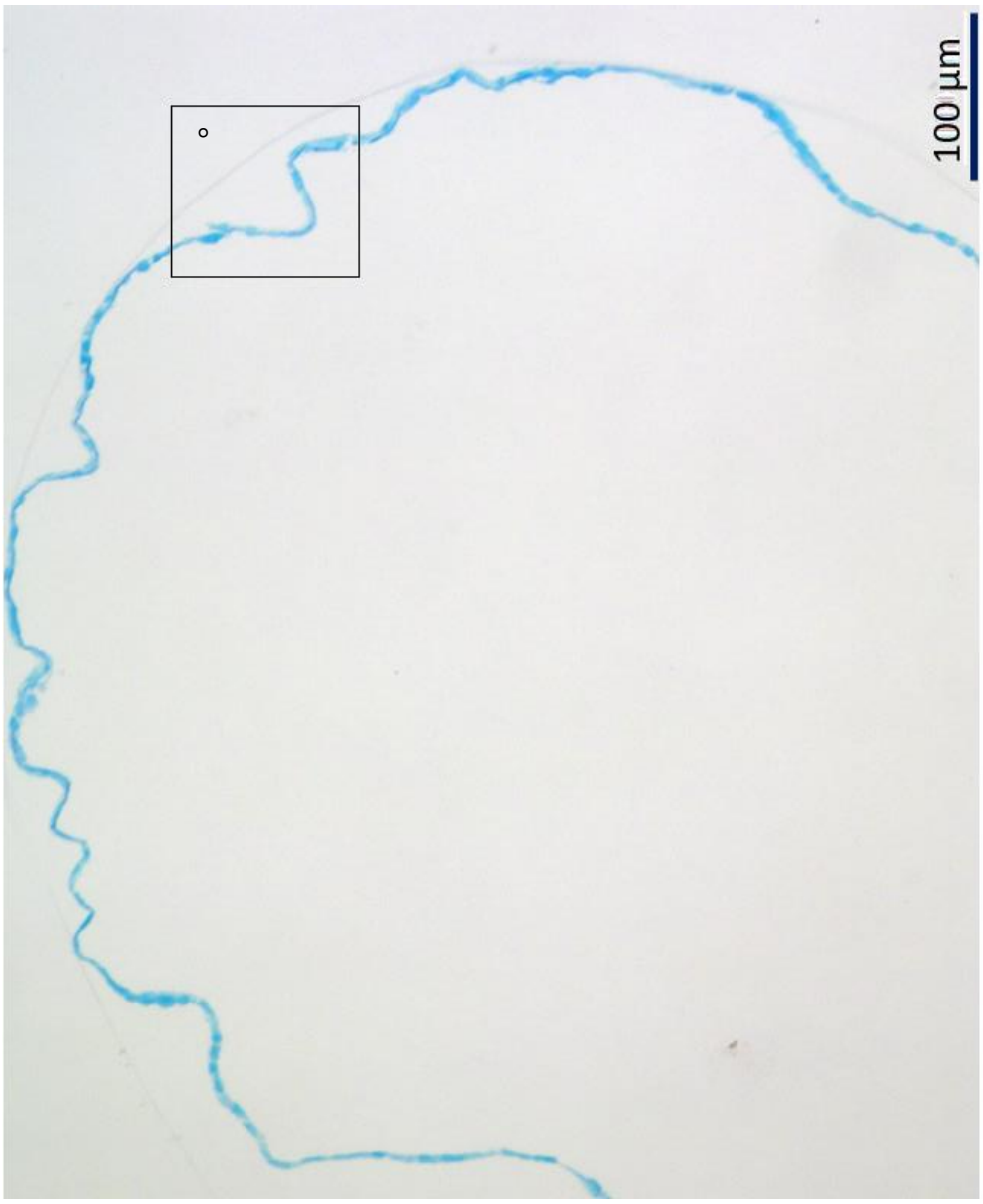
Our first GOAC series were initially seeded with Caco-2 cells only. To visually validate their viability and their aptitude for parietal mucus secretion, direct in-GOAC Alcian blue staining was performed. The same staining was performed on a negative control chip seeded with the same Caco-2 load but with no prior ECM coating of the GOAC, theoretically preventing cell adhesion, growth to confluence and parietal mucus secretion. Light microscopy through the GOAC's fully transparent PDMS and glass scaffold allows for direct upper view of these stained GOAC channels **(Fig. V.2.a-b)**. Furthermore, by performing the in-GOAC Alcian blue staining protocol, we serendipitously discovered that some segments of the stained GOAC's inlet

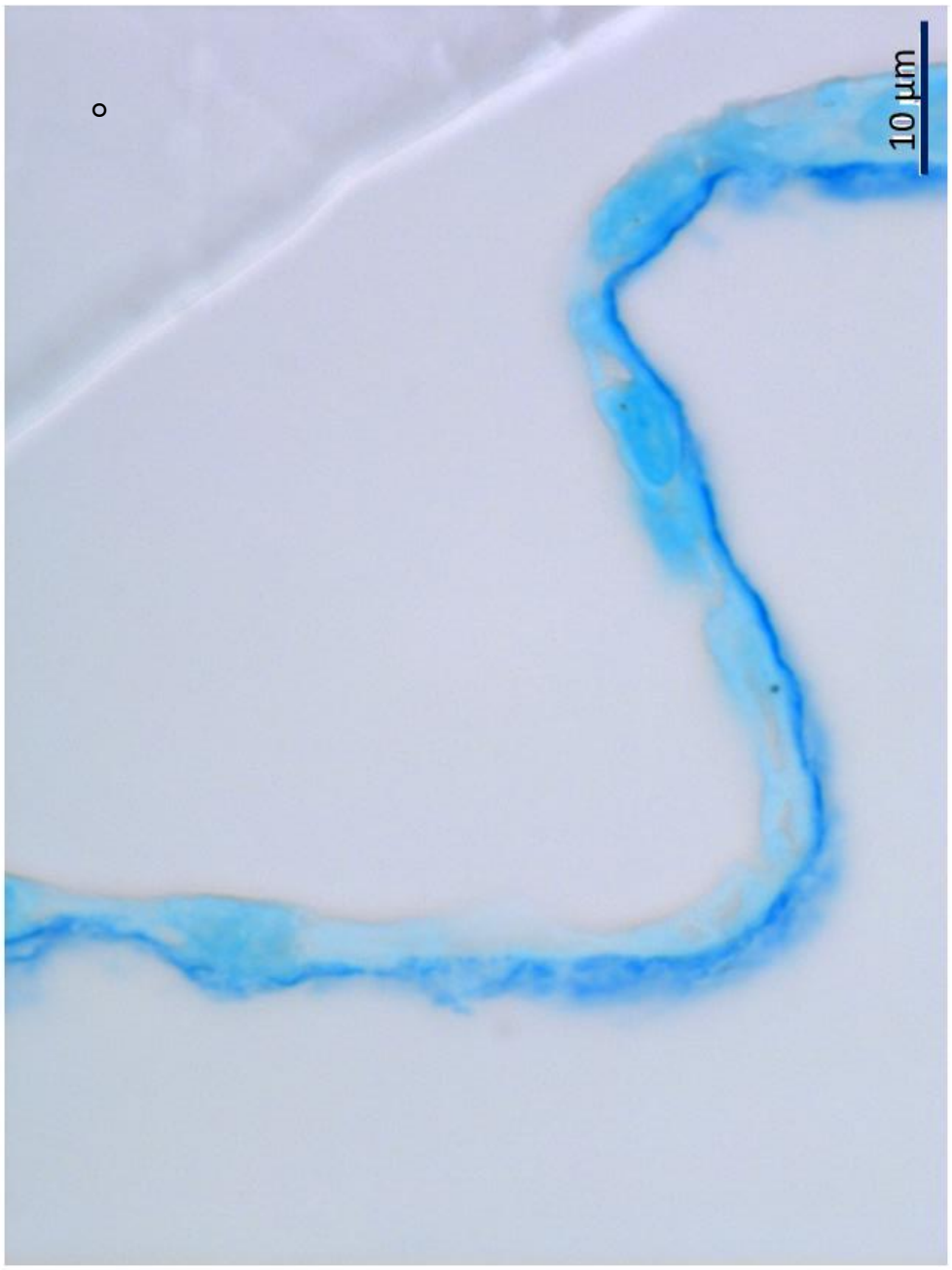
catheters remained similarly stained, and were thus suspected to have been cellularized during the ECM coating and cell seeding process. Whereas the glass-PDMS scaffold of the actual GOAC channel was not suited for microtome sectioning, these catheter segments however were deemed microtome-compatible. To further validate the developed model, transverse histological sections were performed in one of these catheters, confirming the hypothesis that the Caco-2 mono-culture achieved circumferential epithelialization of the catheter's inner walls, displaying a continuous ~2µm thick apical strip of intensely Alcian blue-stained material, likely to be an apical mucus layer **(Fig. V.2.d-e)**.

When ulterior GOACs were instead seeded with a 50%/50% mix of Caco-2 and LS 174T cells ("bicellular GOACs"), we similarly showed the ability of these cell lines to co-exist in cultured GOACs **(Fig. V.2.c)**. To validate the functional maturity of these GOACs, two quantitative ELISA tests specifically targeting mucins MUC2 and MUC5AC respectively were performed on egressing culture medium of three triplicates of GOACs (100% Caco-2 ; 100% LS 174T ; 50%/50% Caco-2 – LS 174T) and on FULL medium as a negative control. Results suggest bicellular GOACs produced an optimal compromise in MUC2-MUC5AC secretion profile compared to mono-cellular counterparts. MUC2 titers were indeed significantly higher in bicellular GOACs than in 100% Caco-2 (univariate ANOVA p-value < 0.001) or 100% LS 174T ($p = 0.01$) GOACs. MUC5AC titers were significantly higher in bicellular GOACs than in 100% Caco-2 GOACs ($p < 0.001$), but lower than in 100% LS 174T GOACs ($p < 0.001$) **(Fig. V.2.f-g)**.



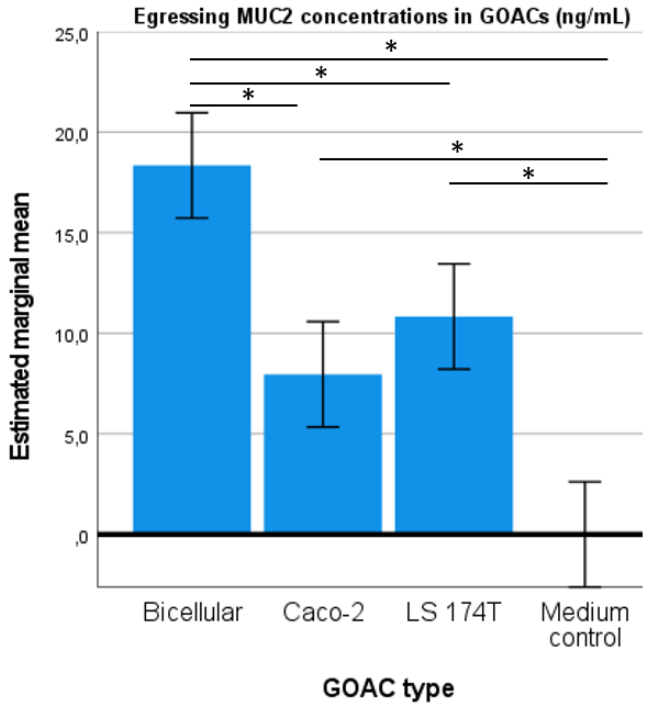
4231


100 µm
d



e

f


Egressing MUC2 concentrations in GOACs (ng/mL)
Estimated marginal mean
25,0
20,0
15,0
10,0
5,0
,0
Bicellular
Caco-2
LS 174T
Medium control
GOAC type

4268

g

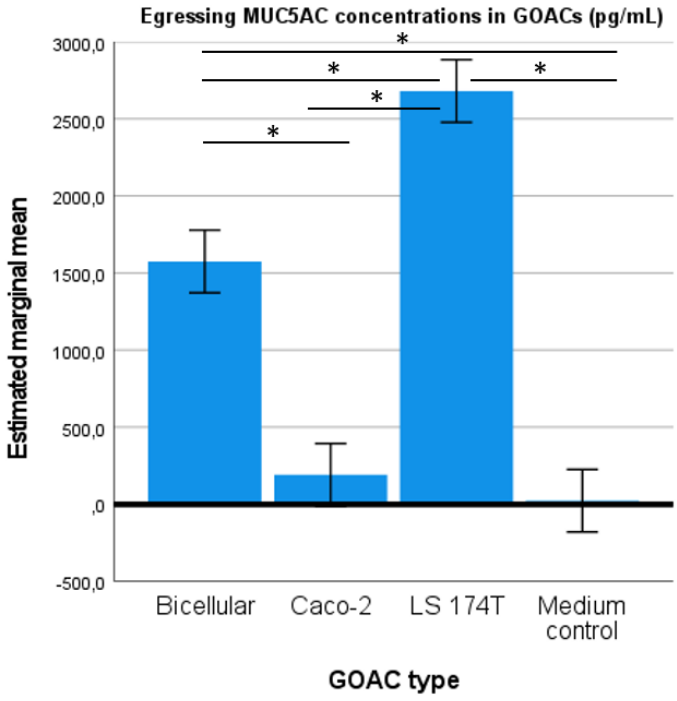

Egressing MUC5AC concentrations in GOACs (pg/mL)
Estimated marginal mean
3000,0
2500,0
2000,0
1500,0
1000,0
500,0
,0
-500,0
Bicellular
Caco-2
LS 174T
Medium control
GOAC type

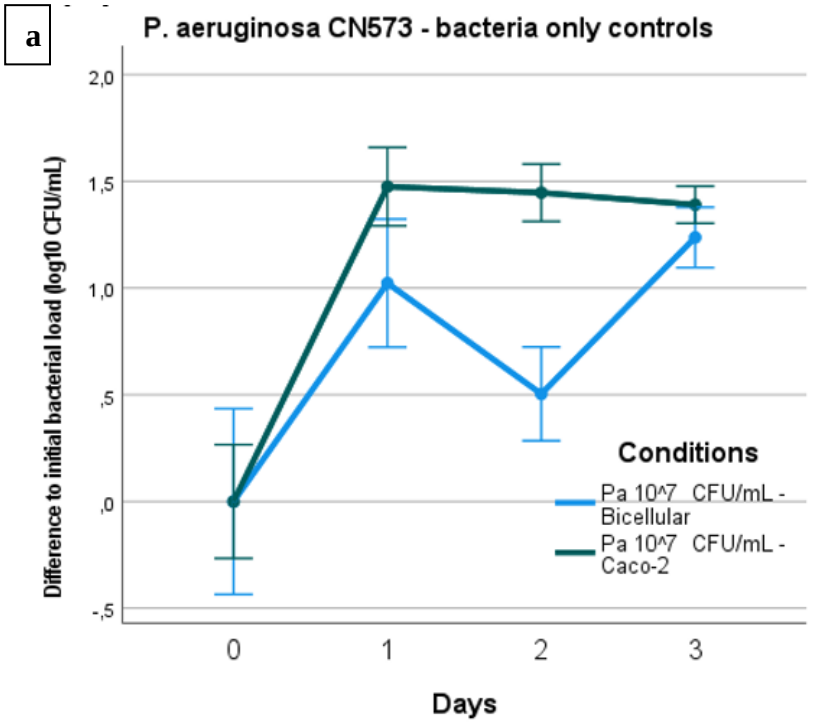
Fig. V.2 | GOAC visualization and mucus production. (a) Upper view in light microscopy of a negative control GOAC (seeded with usual cell load but without prior extra-cellular matrix coating) shows little to no staining after in-GOAC Alcian blue staining protocol compared to a fully-confluent 100% Caco-2 cellularized GOAC **(b)** and a 50%/50% Caco-2 – LS 174T bicellular GOAC **(c)**. General **(d)** and close-up view **(e)** of a histological section performed in a cellularized segment of a 100% Caco-2 GOAC's inlet catheter add further validation of the model's inner tissue architecture. A continuous mono-layer epithelium is seen lining the inner plastic wall (°) of the inlet catheter, seemingly producing a continuous ~2µm-thick layer of an intensely Alcian-blue-stained compound, likely mucus. Quantitative ELISA tests targeting mucin MUC2 **(f)** reveals significantly superior MUC2 production in bicellular GOACs than in both medium negative control and any mono-cellular GOACs. Identical assay targeting mucin MUC5AC **(g)** reveals significantly superior MUC5AC production in bicellular GOACs than in medium negative control and in Caco-2 mono-cellular GOACs, but significantly inferior than in LS 174T mono-cellular GOACs.

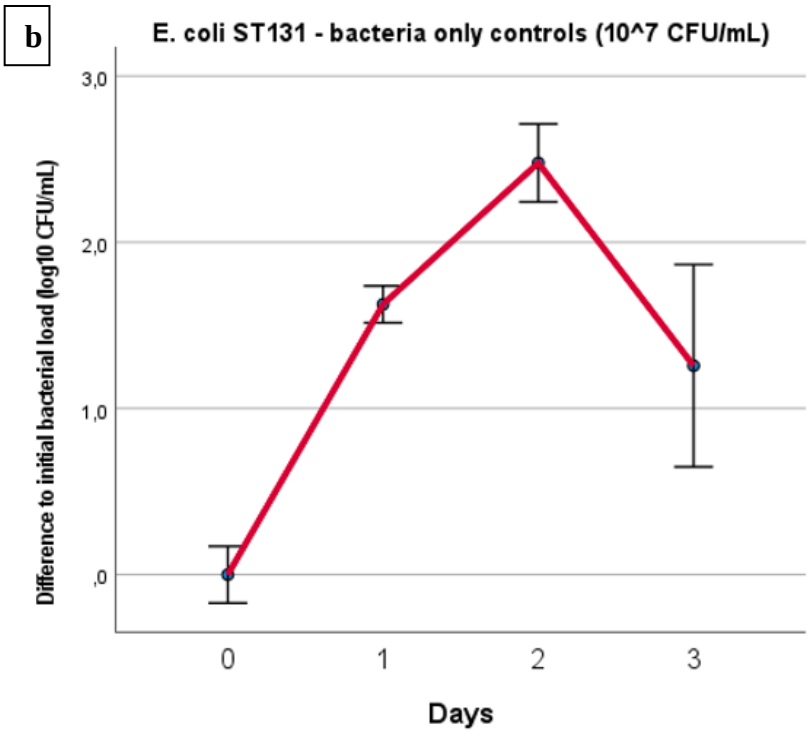
V.4.2 GOAC bacterial infection outcomes

We started our first infection series with *P. aeruginosa* CN573. At this stage, we were still culturing GOAC series seeded either with 100% Caco-2 cells, or with 50%/50% Caco-2 – LS 174T cells. Therefore, several infection conditions tested with *P. aeruginosa* were compared in both GOAC types. This was not the case anymore during our later infection series with *E. coli* ST131, which focused solely on bicellular GOACs.

GOAC infection and outcome follow-up over 72h was performed. When introduced alone at 10^7 CFU/mL, without adding any phage over 72 hours, *P. aeruginosa* and *E. coli* infection in GOACs followed similar dynamics: on a log10 scale, bacterial titer egressing from the GOAC had significantly increased approximately onefold at 24h regardless of tested condition **(Fig. V.3.a-c)**. Bacterial titer then remained stable on a plateau around 10^8 CFU/mL at 48h and 72h for *P. aeruginosa* in 100% Caco-2 GOACs. Comparatively, *P. aeruginosa* knew a temporary decrease in bacterial growth at 48h in bicellular GOACs, but reached similar titers as both other conditions again at 72h **(Fig. V.3.a)**. On the contrary, *E. coli* showed a temporary spike in bacterial growth at 48h. Similar assay with a lower initial bacterial load of 10^5 CFU/mL, used as control conditions for preventive assays, resulted in the same dynamics with a similar 10^8 CFU/mL titer at 24h, remaining stable afterwards **(Fig. V.3.c)**. These series of bacterial infections served as control conditions for phage assays in the next GOAC series.

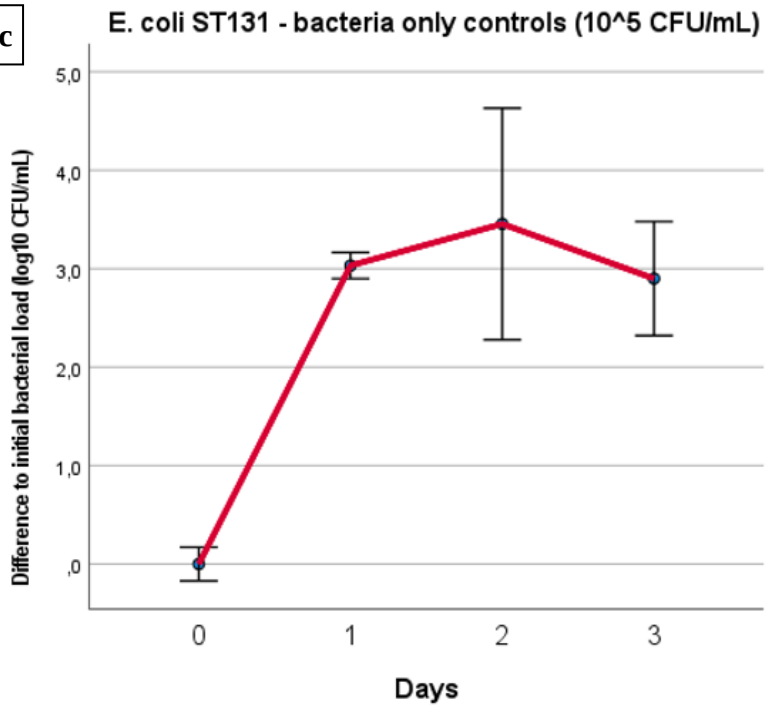
Furthermore, these in-GOAC bacterial infection dynamics seemed similar to those observed over 72h in an OmniLog automated incubator, serving as a control *in vitro* growth condition. Indeed, logarithmic phase of bacterial growth was systematically completed after less than 24h of incubation, then maintaining a stable plateau until final read at 72h **(Fig. V.3.d).**


a
P. aeruginosa CN573 - bacteria only controls
Difference to initial bacterial load (log10 CFU/mL)
2,0
1,5
1,0
,5
,0
-,5
0
1
2
3
Days
Conditions
Pa 10^7 CFU/mL - Bicellular
Pa 10^7 CFU/mL - Caco-2

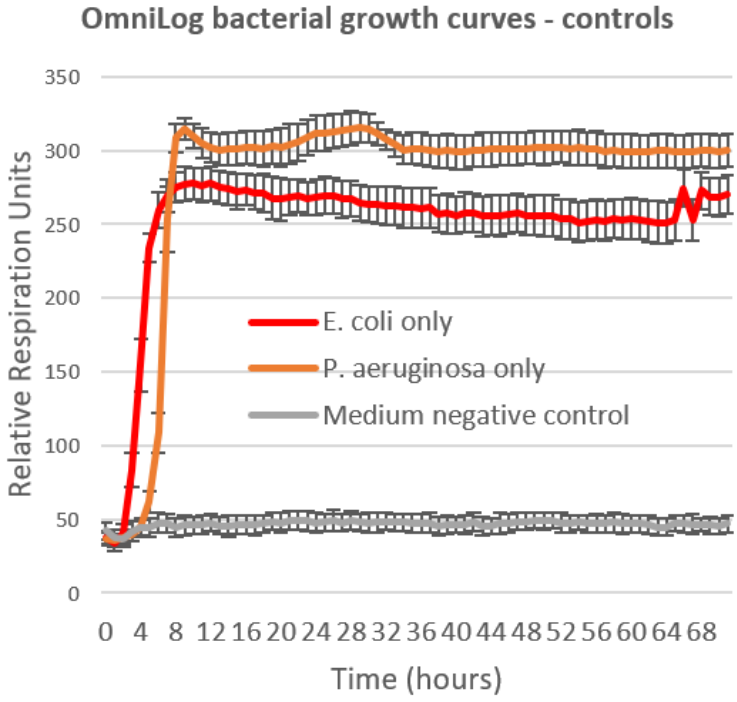

b
E. coli ST131 - bacteria only controls (10^7 CFU/mL)
Difference to initial bacterial load (log10 CFU/mL)
3,0
2,0
1,0
,0
0
1
2
3
Days

4317

c


E. coli ST131 - bacteria only controls (10^5 CFU/mL)
Difference to initial bacterial load (log10 CFU/mL)
5,0
4,0
3,0
2,0
1,0
,0
0
1
2
3
Days

d


OmniLog bacterial growth curves - controls
Relative Respiration Units
350
300
250
200
150
100
50
0
E. coli only
P. aeruginosa only
Medium negative control
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68
Time (hours)

4318

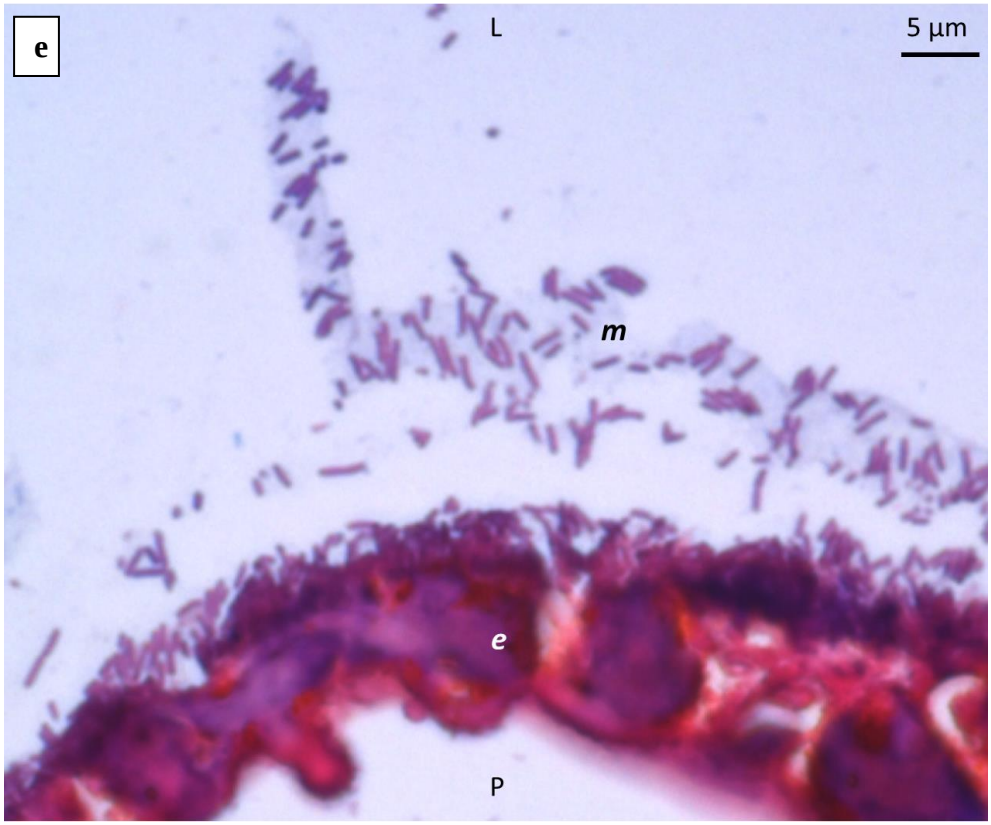


Fig. V.3 | GOAC and OmniLog bacteria-only control conditions. Bacterial titers evolution in GOACs when infected with *P. aeruginosa* CN573 (10^7 CFU/m ; n=3) **(a)** or *E. coli* ST131 at either higher (10^7 CFU/mL ; n=3) **(b)** or lower (10^5 CFU/mL ; n=3) initial titer **(c)**. Bacterial control growth curves in static *in vitro* conditions using an OmniLog automated incubator, bacterial respiration units being measured as a proxy for bacterial growth (n=16 wells for each condition ; initial bacterial load 10^5 CFU/well ; error bars represent +/- 1 standard deviation of the mean) **(d)**. Microscopic view of a GOAC inlet catheter transversal histological section after 48h *P. aeruginosa* CN573 10^7 CFU/mL colonization after Gram staining **(e)**. *P. aeruginosa* appear as numerous typical Gram-negative pink-red bacilli that seemingly

form a homogeneously adherent lining to the apical pole at the continuous Caco-2 GOAC epithelium (*e*), and can also be seen forming a consortium in the likely apical mucus lining (*m*, grey). *[Pa: P. aeruginosa ; CFU: Colony Forming Unit]*

V.4.3 *P. aeruginosa* CN573 versus phage PNM

Joint introduction of both *P. aeruginosa* and its corresponding phage PNM was analyzed under different conditions in both Caco-2 GOACs and bicellular GOACs. The only tested MOI was 1. On at least one out of three timepoints (24, 48 and 72h), all tested conditions differed significantly from aforementioned control GOACs infected with the same *P. aeruginosa* load without phage, though to different extents **(Fig. V.4)**.

P. aeruginosa and PNM were first mixed at MOI 1 immediately before manual instillation in 100% Caco-2 GOACs and restart of the pumping system. This led to a marked reduction in bacterial titer inside the GOACs, all three GOACs of the triplicate reaching below the bacterial detection threshold at 24h ($<10^2$ CFU/mL). One of these two GOACs then reconstituted a detectable bacterial population, eventually surpassing the initial instilled bacterial titer at 72h only. The other two GOACs remained at undetectable bacterial titers for 72h, suggesting sterility **(Fig. V.4.a)**.

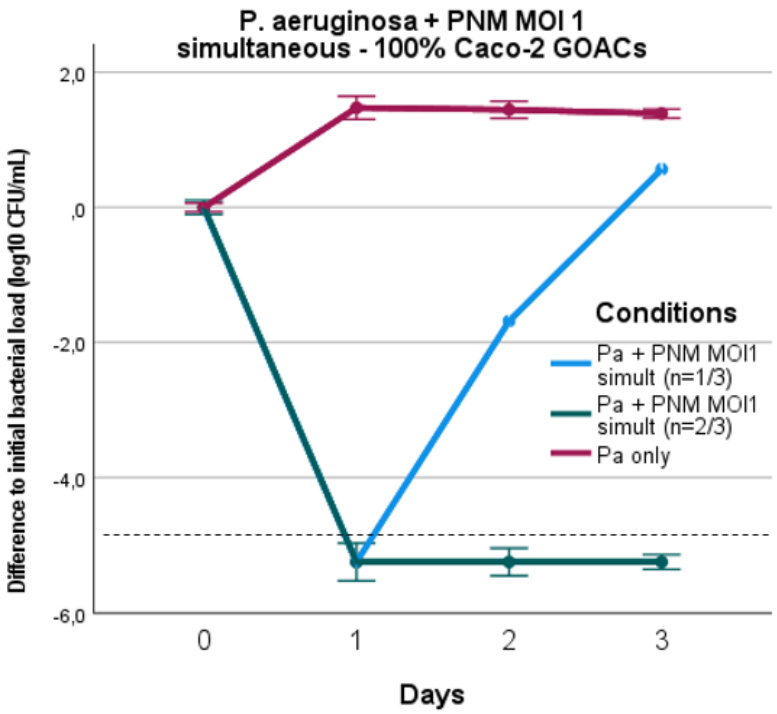
We then introduced these same bacterial and phage load inside a similar triplicate of 100% Caco-2 GOACs, but this time by first instilling *P.*

aeruginosa alone and letting it incubate in the GOACs statically in a 37°C / 5% CO_2 incubator for one hour before introducing the same phage load (MOI = 1). Again, a reduction in bacterial titer was observed in all three GOACs, but to a lesser extent compared to the previous condition of simultaneous administration. Indeed, none of the GOACs reached bacterial undetectability at any timepoint, and all three had grown back to surpass initial bacterial titer at 72h **(Fig. V.4.b)**.

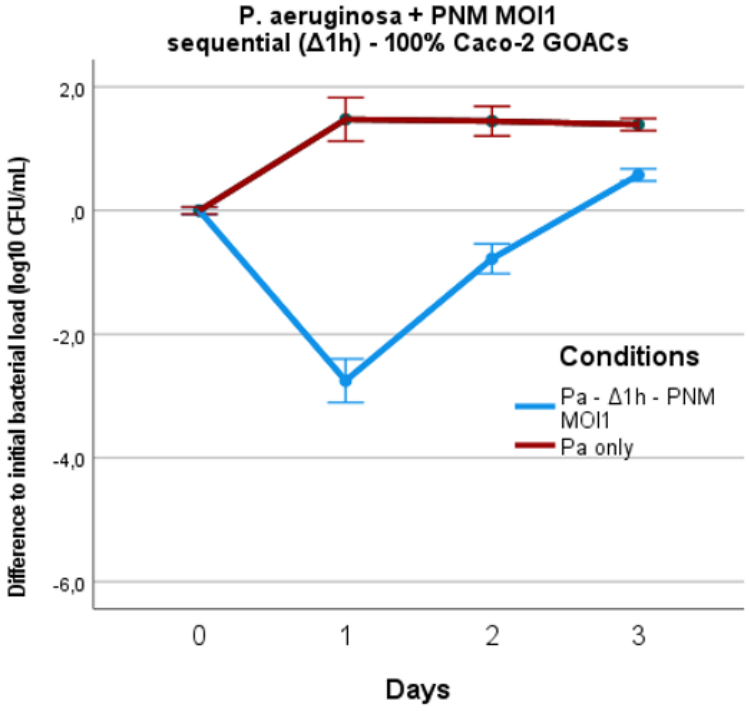
Replicating both these conditions in bicellular GOACs instead of Caco-2 GOACs led to even more bacterial-favoring outcomes, all other things being equal. Simultaneous phage-bacteria introduction again led to significant reduction in bacterial titer at 24h, but no GOAC ever reached bacterial undetectability, contrasting with their 100% Caco-2 counterparts. In addition, bacterial titer had surpassed initial instilled titer at 48h. Sequential administration after a similar one hour delay after bacterial incubation was this time unable to induce any reduction in initial instilled bacterial titer at 24h. At 72h, both of these conditions' bacterial titers had reached similar levels as their bacteria-only control GOACs **(Fig. V.4.c)**.

During these assays, bacterial growth or regrowth despite phage lytic pressure was accompanied by the emergence of phenotypic variants of *P. aeruginosa*, identified on visual basis of modified colony morphology on blood agar plates **(Fig. V.4.e-f)**.

a

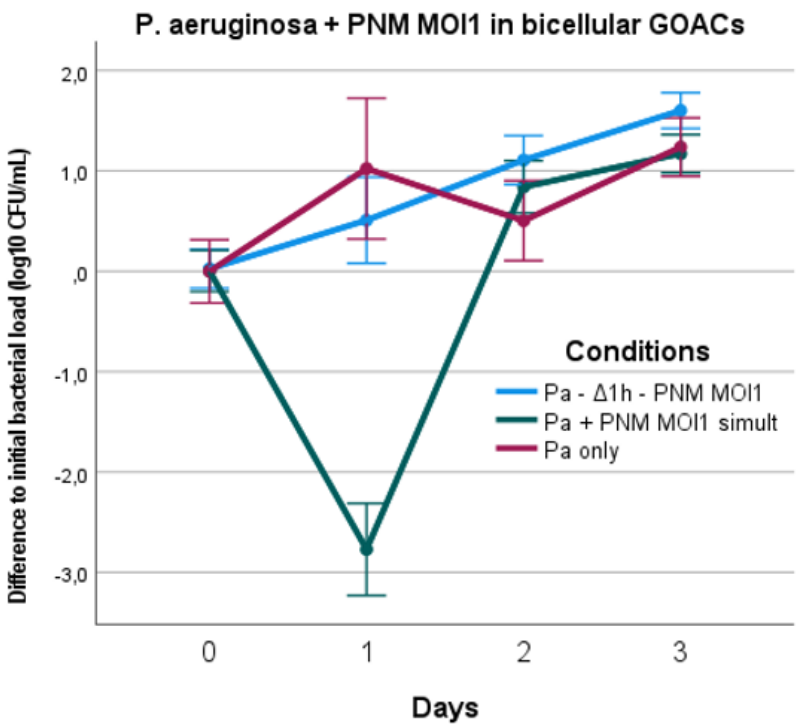

P. aeruginosa + PNM MOI 1
simultaneous - 100% Caco-2 GOACs
Difference to initial bacterial load (log10 CFU/mL)
2,0
,0
-2,0
-4,0
-6,0
0
1
2
3
Days
Conditions
Pa + PNM MOI1 simult (n=1/3)
Pa + PNM MOI1 simult (n=2/3)
Pa only

b

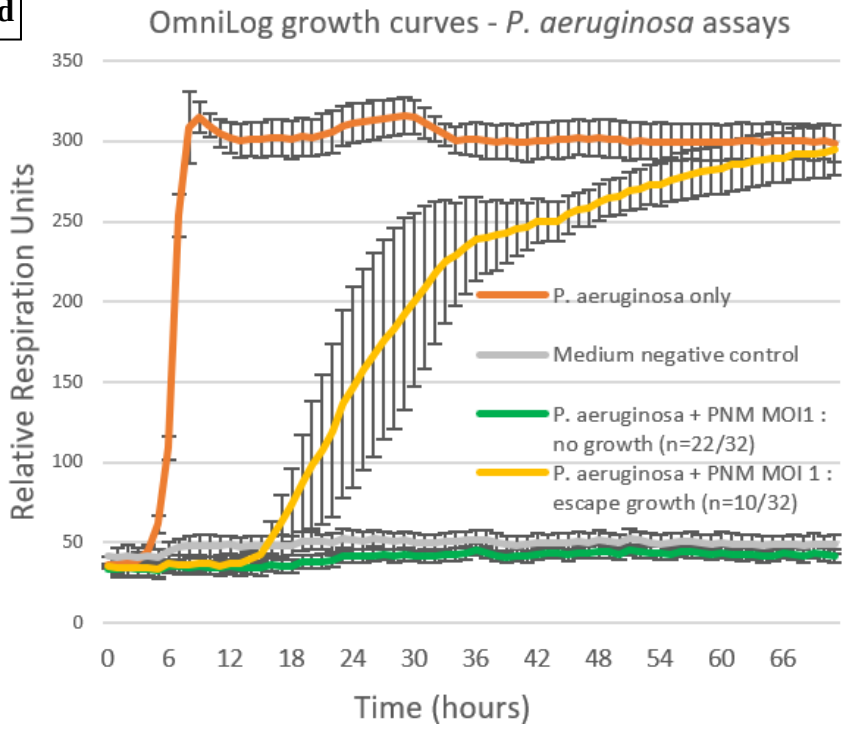

P. aeruginosa + PNM MOI1
sequential (Δ1h) - 100% Caco-2 GOACs
Difference to initial bacterial load (log10 CFU/mL)
2,0
,0
-2,0
-4,0
-6,0
0
1
2
3
Days
Conditions
Pa - Δ1h - PNM MOI1
Pa only

4375

c

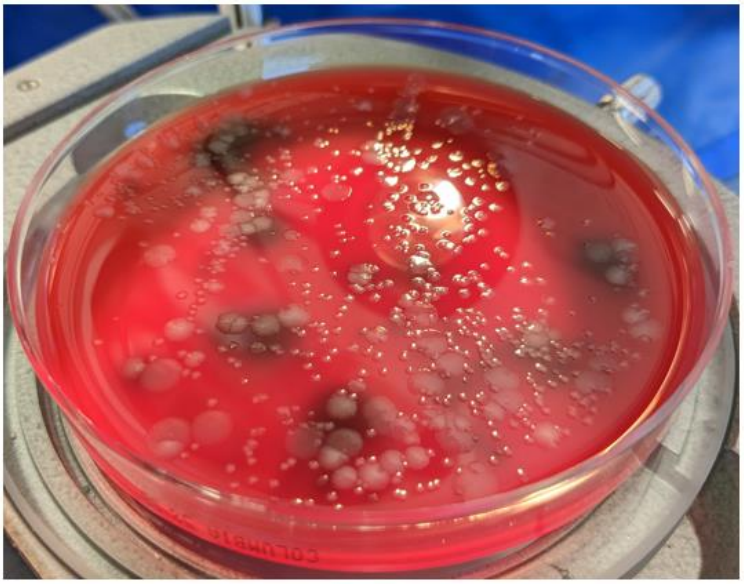

P. aeruginosa + PNM MOI1 in bicellular GOACs
Difference to initial bacterial load (log10 CFU/mL)
2,0
1,0
,0
-1,0
-2,0
-3,0
0
1
2
3
Days
Conditions
Pa - Δ1h - PNM MOI1
Pa + PNM MOI1 simult
Pa only

d

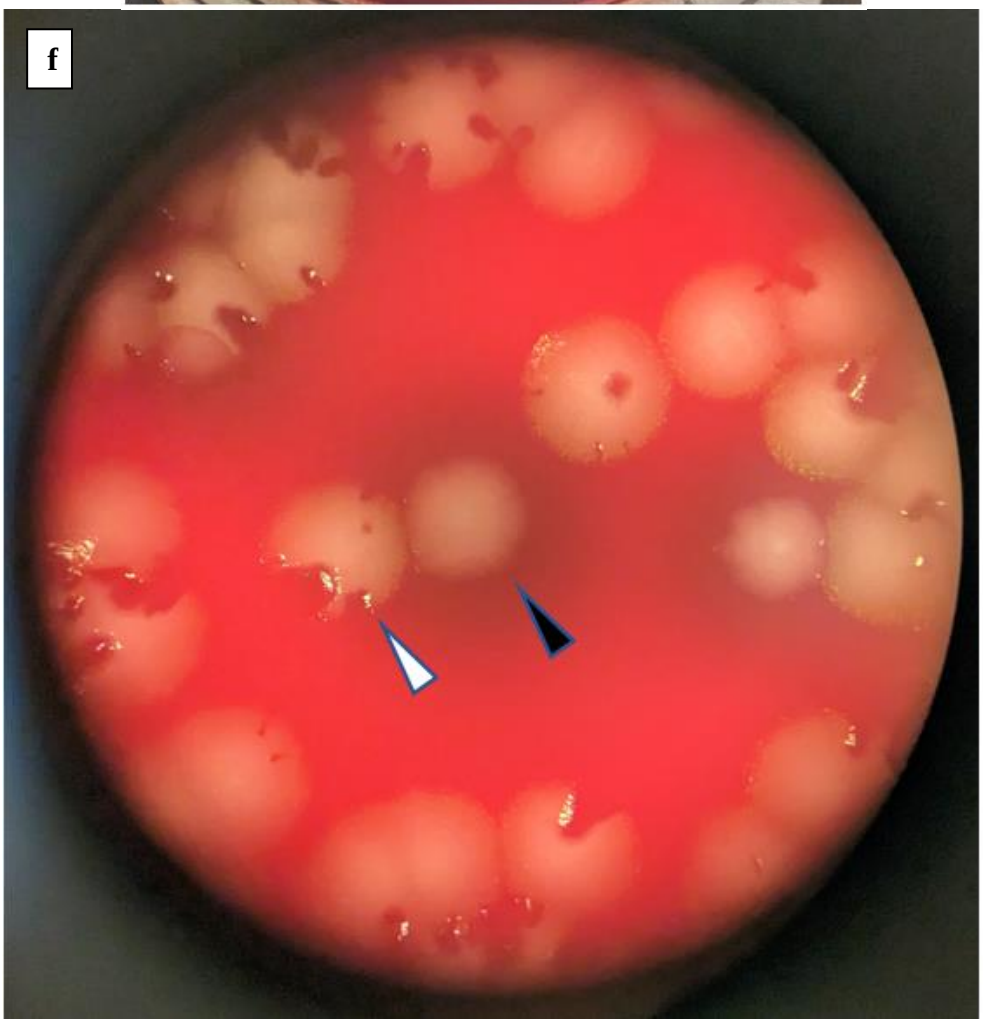

OmniLog growth curves - P. aeruginosa assays
Relative Respiration Units
350
300
250
200
150
100
50
0
0
6
12
18
24
30
36
42
48
54
60
66
Time (hours)
P. aeruginosa only
Medium negative control
P. aeruginosa + PNM MOI1 : no growth (n=22/32)
P. aeruginosa + PNM MOI 1 : escape growth (n=10/32)

4376

e



f



4377

Fig. V.4 | *P. aeruginosa* CN573 versus phage PNM assays. *P. aeruginosa* titers evolution when introduced in GOACs with phage PNM (MOI = 1) in various conditions: **(a)** simultaneous introduction of *P. aeruginosa* and PNM in 100% Caco-2 GOACs (the dotted line representing detection threshold in spread plating, here $<10^2$ CFU/mL) (n=3), **(b)** sequential introduction of *P. aeruginosa* first with subsequent introduction of phage PNM after a 1h static incubation delay in 100% Caco-2 GOACs (n=3), **(c)** replication of these two conditions in bicellular GOACs (n=3 each). **(d)** *P. aeruginosa* and PNM (MOI = 1) control growth curves in static *in vitro* conditions using an OmniLog automated incubator, bacterial respiration units being measured as a proxy for bacterial growth (initial bacterial load 10^5 CFU/well ; n = 32 wells for *P. aeruginosa* + PNM, 16 wells for *P. aeruginosa* only and 16 wells for negative control ; error bars represent +/-1 standard deviation of the mean). **(e)** Spread plating on 5% sheep blood Columbia agar plates highlights intense phenotypic diversification in *P. aeruginosa* CN573 after co-evolution with phage PNM in GOACs, here displaying at least four distinct colony morphologies out of a single GOAC after 48h. **(f)** Close-up view of one of these plates reveals co-existence of wild type colonies (white arrow), systematically including phage-induced lysis plaques, along with modified colonies for example exhibiting a dark pigmented halo (black arrow), systematically devoid of any lysis plaques. *[Pa: P. aeruginosa ; GOAC: Gut-On-A-Chip ; MOI: Multiplicity Of Infection]*

All of these variants were confirmed to belong to the *P. aeruginosa* species by MALDI-TOF. These variants' morphotypes were reproductible between GOAC replicates in which both *P. aeruginosa* and PNM were introduced, but not in any of the bacteria-only control GOACs. This led us to suspect a non-random selection of a significant fitness advantage correlated to these phenotypic alterations, including for example the *de novo* acquisition of BPR mechanisms, allowing for bacterial regrowth. To test this hypothesis, these variants were subcultured on blood agar plates and phage PNM's EOP was assessed anew on each of them, compared to the initial *P. aeruginosa* CN573 strain. Results confirmed the emergence of BPR in all variants **(Table V.1)**.

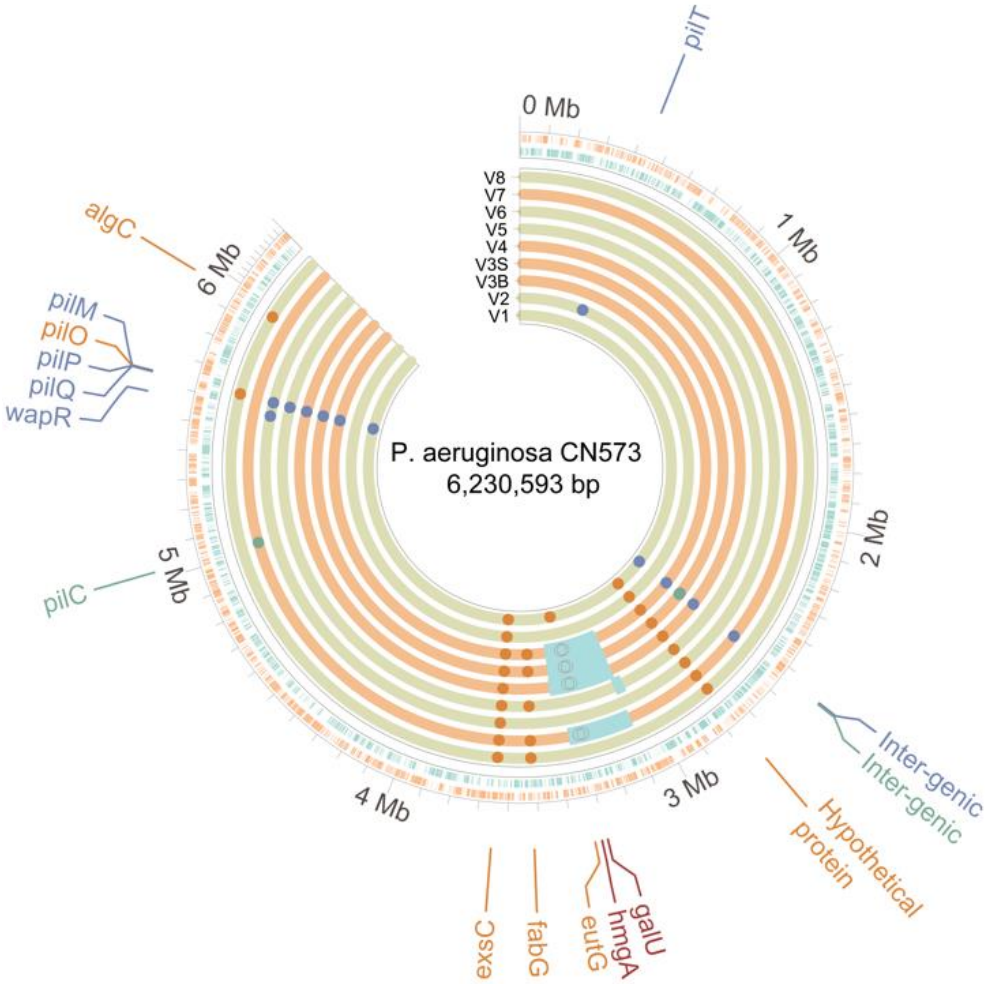
These bacterial variants were then sequenced to investigate the potential genomic basis for these phenotypic modifications. Consistent with the identification of total or partial BPR phenotype for each of them, genomic variations likely to cause these modified phenotypes were identified for each of them. All nine variants sequenced showed a variety of SNPs and indels in genes coding for *P. aeruginosa*'s T4P complex *(pilC, pilM, pilO, pilP, pilQ, pilT)*, known to be a key surface receptor for phage PNM's infectivity and already identified as a BPR driver in clinical cases of anti-*P. aeruginosa* PT[140, 266] **(Table V.1, Fig. V.5)**. Some of these variants also showed other SNPs and indels in genes of other complexes such as the type III secretion system *(exsC)* as well as membrane lipids and LPS biosynthesis (*algC, fabG, wapR)*. A synonymous variant in a putative protein located next to (and suspected to be part of) the type VI secretion systems (T6SS) (MLIPEN_12965) was also detected. Lastly, five variants contained large chromosomal deletions ranging from 46 kb to 330 kb, including four brown-

colored variants which were systematically and specifically found to harbor structural chromosomal deletions including the *galU* and *hmgA* genes **(Table V.1, Fig. V.5)**.

We also tried to compare the rate of emergence of these bacterial growth under phage pressure to a more neutral *in vitro* assay. *P. aeruginosa* CN573 and phage PNM were thus incubated in 96-well microplates in an automated OmniLog incubator, measuring bacterial growth every 20 minutes by tetrazolium dye reduction assay. For the sake of comparison to GOAC conditions, MOI, temperature and duration of incubation were the same (1, 37°C and 72h respectively). Bacterial "escape" growth despite phage pressure was less frequent in these OmniLog assays than in GOACs, most OmniLog replicates (22/32) showing no detectable growth during 72h **(Fig. V.4.d)**. This differs significantly from bicellular GOACs dynamics in both simultaneous and sequential infection conditions (Fischer's exact test p-value = 0.0437), and from Caco-2 GOACs in sequential infection condition (p = 0.0437), but does not differ from Caco-2 GOACs in simultaneous infection condition (p = 1).

Isolate	Colony phenotype	PNM EOP	Phage PNM susceptibility	Mutations in genes	Structural variation
CN573-V1	Bright green big colonies	0,0009	Partly resistant	Frameshift variant in *pilM*, stop gained in *exsC*, missense variant in *eutG*	
CN573-V2	Bright green big colonies	0,0010	Partly resistant	Inframe deletion in *pilT*, stop gained in *exsC*	
CN573-V3B	Brown big colonies	0	Fully resistant	Frameshift variant in *pilQ*, stop gained in *exsC*, missense variant in *fabG*	Chromosomal deletion (including *galU* and *hmgA*)
CN573-V3S	Brown small colonies	0	Fully resistant	Frameshift variant in *pilQ*, stop gained in *exsC*, missense variant in *fabG*,	Chromosomal deletion (including *galU* and *hmgA*)
CN573-V4	Brown small colonies	0	Fully resistant	Frameshift variant in *pilP*, stop gained in *exsC*,	Chromosomal deletion (including *galU* and *hmgA*)
CN573-V5	Bright green big colonies	0	Fully resistant	Frameshift variant in *pilQ*, stop gained in *exsC*, missense variant in *fabG*	Chromosomal deletion
CN573-V6	Pale green small colonies	0	Fully resistant	Frameshift variant in *pilP and wapR*, stop gained in *exsC*,	
CN573-V7	Brown small colonies	0	Fully resistant	Frameshift variant in *pilC*, stop gained in *exsC*, and missense variant in *fabG*	Chromosomal deletion (including *galU* and *hmgA*)
CN573-V8	Pale green irregular colonies	0	Fully resistant	Stop gained in *exsC* and *pilO*, missense variants in *fabG* and *algC*	

Table V.1 | *P. aeruginosa* variants typing. Summary of phage susceptibility testing and genomic characterization of 9 variants of *P. aeruginosa* CN573 retrieved after co-evolution with phage PNM in GOACs.



4453

4454

4455 **Fig. V.5 | Circular chromosomic view (CCV) of the bacterial genomes of**
4456 **the nine sequenced *P. aeruginosa* variants.** The first two rings (starting
4457 from the outside to the inside) indicate the coding regions on the plus and
4458 minus strands, and the nine inner rings show the genomic variations in the
4459 partially phage PNM-resistant V1 and V2 isolates and the fully resistant V3B,
4460 V3S, V4, V5, V6, V7 and V8 isolates. Orange inner rings indicate the […]

[…] four brown-colored variants. Small deletions are highlighted in dark blue, insertions in green and SNPs in orange. The light blue boxes highlight the large chromosomal deletions detected in some of the variants. The position of the *galU* and *hmgA* genes, associated with the brown phenotype, are shown in burgundy. [*bp: basepairs ; Mb: megabases ; SNP: single-nucleotide polymorphism]*

V.4.4 *E. coli* ST131 versus phages ES17 and HP3

Similar assays were conducted to investigate infection dynamics between *E. coli* ST131 and the two anti-*E. coli* lytic phages ES17 and HP3. For these *E. coli* assays, only bicellular GOACs were used.

E. coli was first mixed at MOI 1 with phage ES17 or phage HP3 immediately before manual instillation in bicellular GOACs and restart of the pumping system. Compared to bacteria-only control GOACs, this only induces a statistically significant reduction in bacterial titer at 24h with HP3, not with ES17. At 48h, both ES17- and HP3-treated GOACs have lower bacterial titer than controls, though both are also significantly higher than initially instilled bacterial titer. At 72h, both conditions had bacterial titers similar or superior to control GOACs (**Fig. V.6.a**).

We then introduced these same bacterial and phage load inside a similar triplicate of bicellular GOACs, but this time by first instilling *E. coli* alone and letting it incubate in the GOACs statically in a 37°C / 5% CO_2

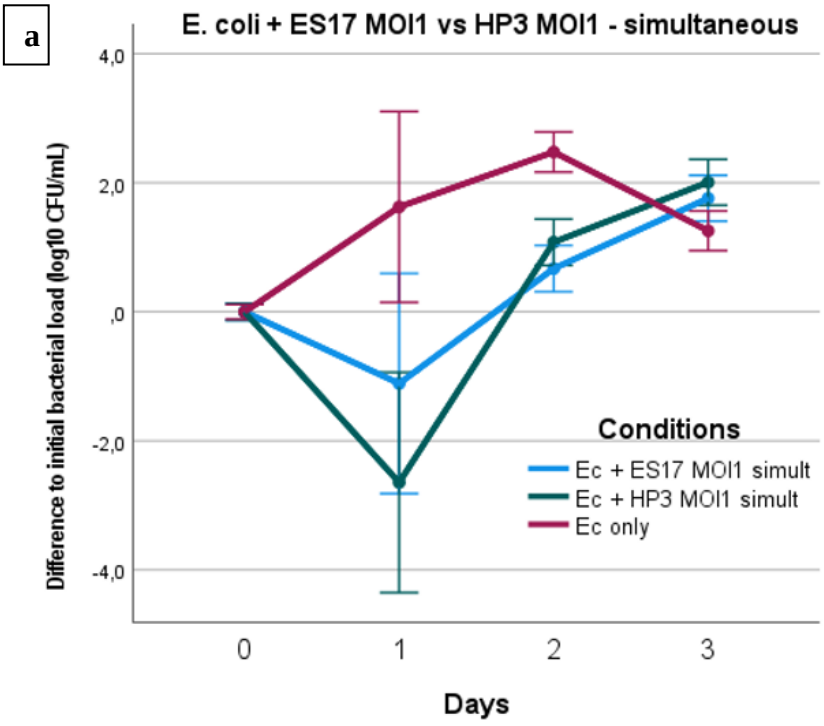
incubator for one hour before introducing the same phage load (MOI 1) of either ES17 or HP3. Similar to the *P. aeruginosa* assays, this led to more bacteria-favoring outcomes, all other things being equal. HP3-induced relative reduction in bacterial titer at 24h, while still statistically significant, was less marked. ES17 failed to even maintain *status quo* compared to initially instilled bacterial titer at 24h. By 72h, both conditions had reached similar bacterial titers to bacteria-only controls (**Fig. V.6.b**).

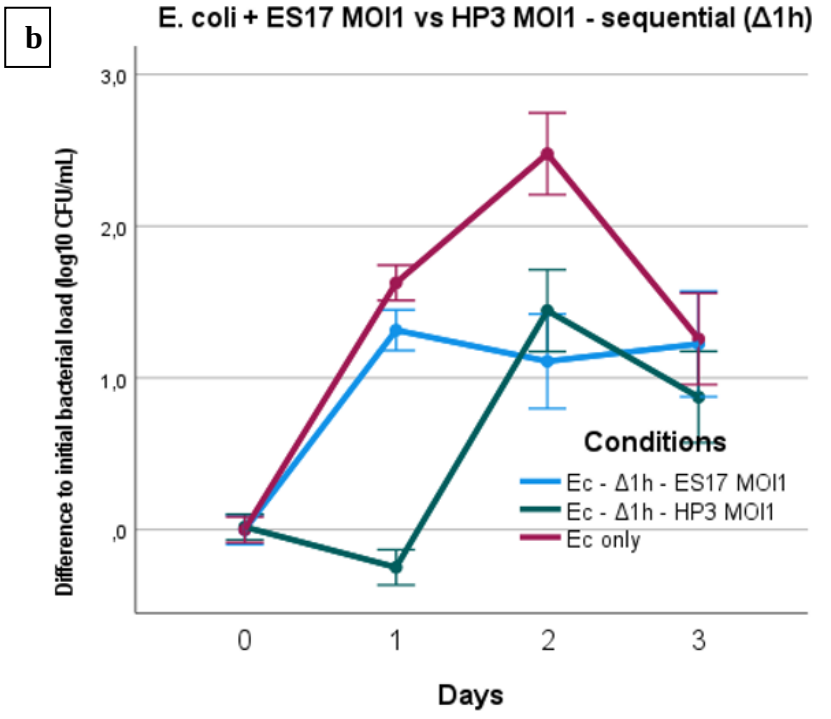
We decided to replicate this condition of sequential administration using a tenfold higher phage load (MOI 10 instead of 1 for all previous assays), keeping all other parameters unchanged. This led to moderately more favorable phage effect, seemingly reverting the bacteria-favoring effect of sequential instead of simultaneous administration. Titer curves are indeed largely similar in these conditions to those of the GOACs tested with simultaneous bacteria-phage introduction. Yet, even this higher MOI failed to elicit major reduction of bacterial titers below detectability threshold at any timepoint. In addition, ES17 again seemed to perform more poorly than HP3 in these conditions, failing to reduce bacterial titer below initially instilled bacterial titer at any timepoint (**Fig. V.6.c**).

We then investigated whether phage efficacy in these GOACs could be improved by administering them in a continuous way, instead of a single initial introduction of a given phage load. To test this, syringes loaded with NAB medium in the alimentation pump were supplemented with MOI 10 titers of either ES17 or HP3, ensuring continuous top-down infusion of both phages through the whole length of the model for the whole 72h of the assay. This did not lead to significantly better phage outcomes, bacterial titers never

reaching sub-detectability levels at any timepoint, surpassing initially instilled bacterial titer at 48h and even slightly surpassing bacteria-only control titer at 72h. However, for the first time, ES17 seemingly performed slightly better than HP3 at 24h, achieving significantly lower bacterial titer than bacteria-only control, as opposed to HP3 (**Fig. V.6.d**).

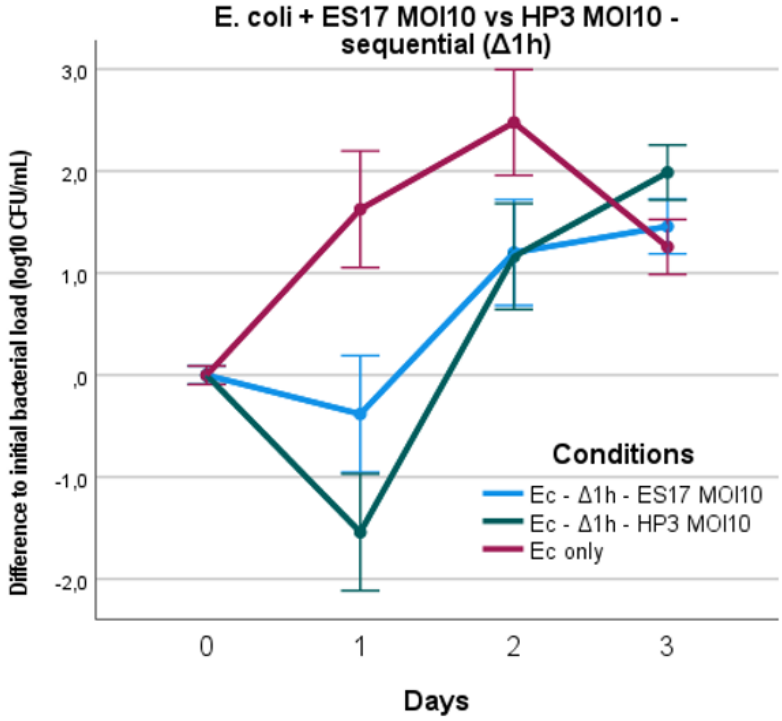
In the next assays, we wanted to investigate the potential preventive properties of these two phages rather than their curative ones. As opposed to the previously investigated "sequential" condition where bacteria introduction precedes phage introduction, here, we pre-treated bacteria-free GOACs with phages, to then introduce bacteria and analyze if phage pre-treatment could prevent bacterial settlement in the GOACs. GOACs were pre-treated with phages then washed with two different washing protocols (see Material and Methods section). Bacterial introduction to the model was then attempted by infusing *E. coli* in a continuous flow, similar to the previous assay: syringes loaded with NAB medium in the alimentation pump were supplemented with a lower titer of 10^5 CFU/mL, which were then infused through the GOACs at a usual rate of 120µL/h. Control GOACs in these conditions were thus also seeded with 10^5 CFU/mL of *E. coli* instead of the usual 10^7 CFU/mL in all other GOAC assays. Despite evidence of phage persistence in the pre-treated GOACs by observance of lysis plaques on output CFU in all four conditions, neither of these pre-treatment protocols managed to prevent bacterial settlement and growth in these GOACs, none of the bacterial titers measured being inferior to their bacteria-only controls at any timepoint (**Fig. V.6.e-f**).


a
E. coli + ES17 MOI1 vs HP3 MOI1 - simultaneous
Difference to initial bacterial load (log10 CFU/mL)
4,0
2,0
,0
-2,0
-4,0
0
1
2
3
Days
Conditions
Ec + ES17 MOI1 simult
Ec + HP3 MOI1 simult
Ec only

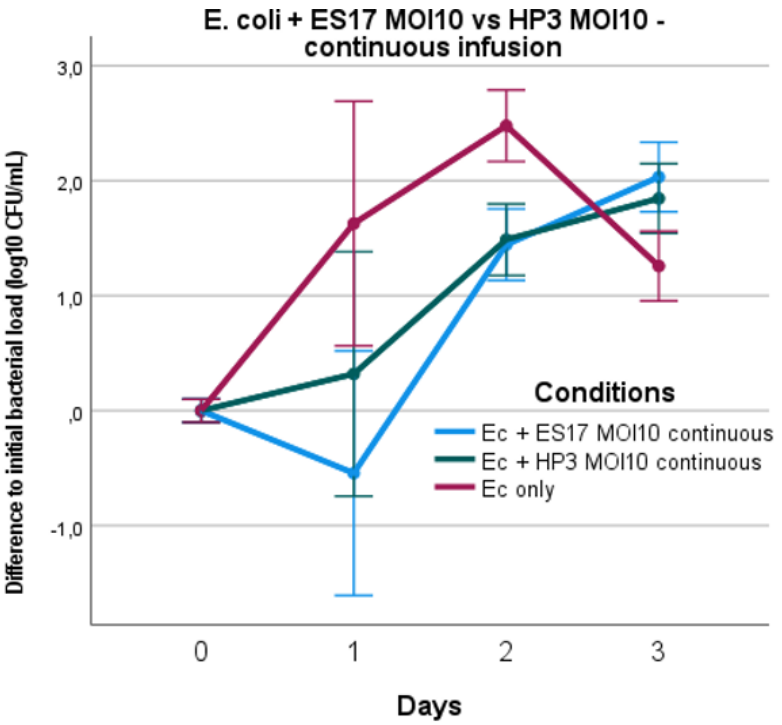

b
E. coli + ES17 MOI1 vs HP3 MOI1 - sequential (Δ1h)
Difference to initial bacterial load (log10 CFU/mL)
3,0
2,0
1,0
,0
0
1
2
3
Days
Conditions
Ec - Δ1h - ES17 MOI1
Ec - Δ1h - HP3 MOI1
Ec only

4534

c

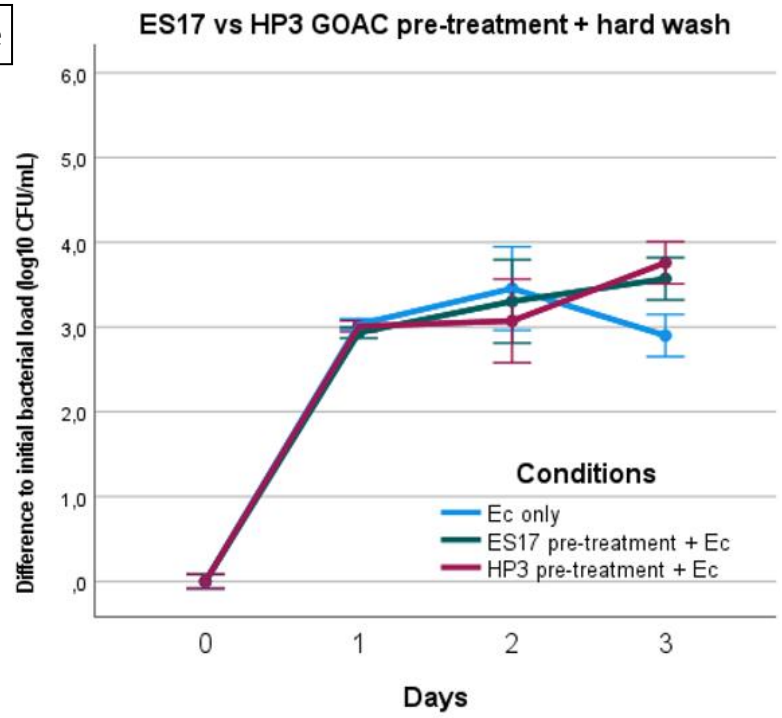

E. coli + ES17 MOI10 vs HP3 MOI10 - sequential (Δ1h)
Difference to initial bacterial load (log10 CFU/mL)
3,0
2,0
1,0
,0
-1,0
-2,0
0
1
2
3
Days
Conditions
Ec - Δ1h - ES17 MOI10
Ec - Δ1h - HP3 MOI10
Ec only

d

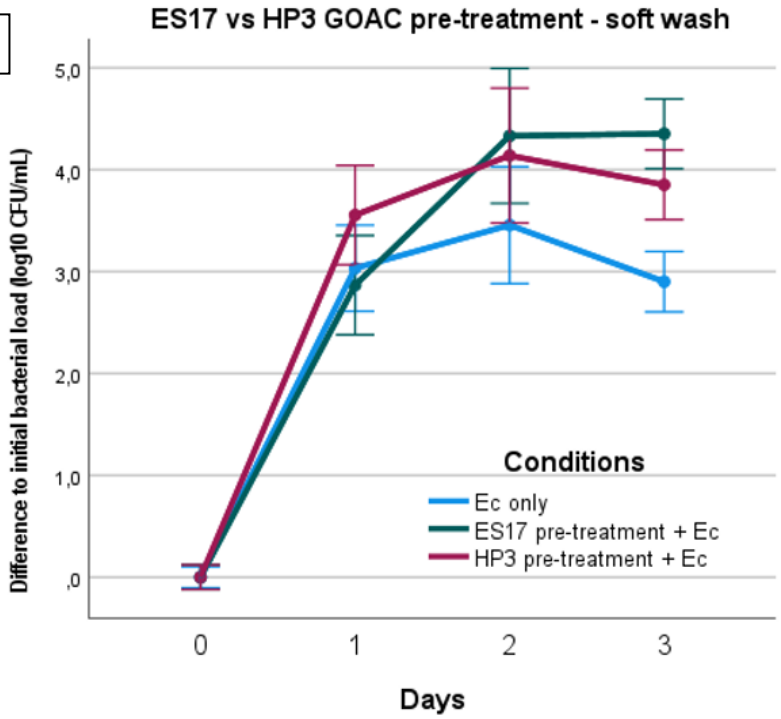

E. coli + ES17 MOI10 vs HP3 MOI10 - continuous infusion
Difference to initial bacterial load (log10 CFU/mL)
3,0
2,0
1,0
,0
-1,0
0
1
2
3
Days
Conditions
Ec + ES17 MOI10 continuous
Ec + HP3 MOI10 continuous
Ec only

4535

e


ES17 vs HP3 GOAC pre-treatment + hard wash
Difference to initial bacterial load (log10 CFU/mL)
6,0
5,0
4,0
3,0
2,0
1,0
,0
0
1
2
3
Days
Conditions
Ec only
ES17 pre-treatment + Ec
HP3 pre-treatment + Ec

f


ES17 vs HP3 GOAC pre-treatment - soft wash
Difference to initial bacterial load (log10 CFU/mL)
5,0
4,0
3,0
2,0
1,0
,0
0
1
2
3
Days
Conditions
Ec only
ES17 pre-treatment + Ec
HP3 pre-treatment + Ec

4536

Fig. V.6 | *E. coli* ST131 versus phage ES17 or phage HP3 assays. *E. coli* titers evolution when introduced in bicellular GOACs with either phage ES17 or phage HP3 in various conditions: **(a)** simultaneous introduction of *E. coli* and either phage at MOI = 1 (n=3 each), **(b)** sequential introduction of *E. coli* first with subsequent introduction of either phage after a 1h static incubation delay at MOI = 1 (n=3 each), **(c)** sequential introduction of *E. coli* first with subsequent introduction of either phage after a 1h static incubation delay at MOI = 10 (n=3 each), **(d)** introduction of *E. coli* then submitted to a continuous 72h infusion of either phage at MOI = 10 (n=3 each). Preventive assays were also performed to establish *E. coli* titers evolution when introduced (at a lower initial titer of 10^5 CFU/mL instead of usual 10^7 CFU/mL) in bicellular GOACs previously pre-treated with either phage at titers of 10^8 PFU/mL by letting the phage pre-treatment incubate statically in GOACs for 1h then submitting GOACs to either a hard **(e)** or soft **(f)** washing protocol before *E. coli* introduction (n=3 each). *[Ec: E. coli ; GOAC: Gut-On-A-Chip ; MOI: Multiplicity Of Infection]*

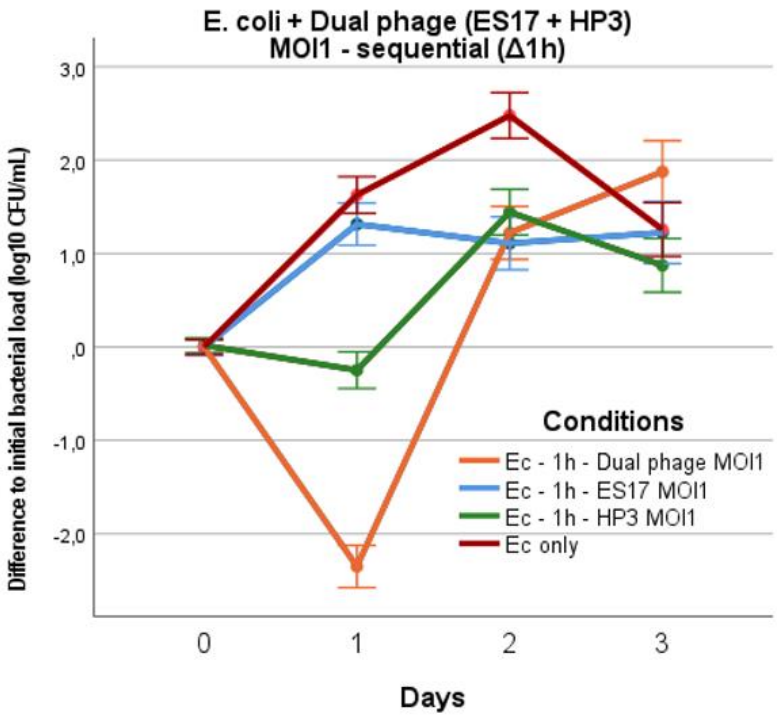
Up to this point, ES17 and HP3 phages had only been tested separately, aiming to compare their respective efficacy, or lack thereof, at modifying in-GOAC bacterial dynamics. Next, we wanted to investigate whether these phages seemed to have synergistic potential in this model. Accordingly, we replicated the sequential administration series by first incubating *E. coli* alone in GOACs, then introducing a 50%/50% mix of ES17 and HP3 phages (combined MOI of 1 and 10 respectively). At MOI 1, this assay generated significantly better phage outcomes at 24h than either mono-

phage MOI 1 counterparts in the same conditions. This was not the case at combined MOI 10, which unexpectedly performed worse than MOI 1 at 24h, though still managing to temporarily reduce bacterial titer below initially instilled bacterial titer, but not to a greater extent than HP3 or ES17 alone at the same MOI 10 (**Fig. V.7.a-b**).

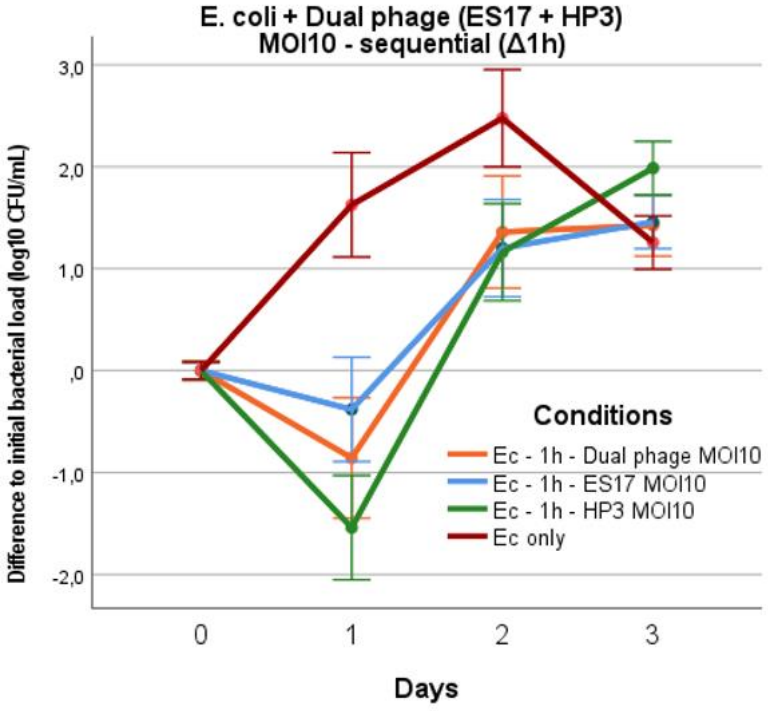
Following a similar approach to the *P. aeruginosa* assays, development of thriving bacterial growth under various conditions of phage pressure in these GOACs led us to investigate potential underlying causes. In multiple independent GOACs, the occasional observation of phenotypic modifications in *E. coli* colony morphologies led us to postulate again a fitness advantage in these variants, possibly correlated to BPR acquisition (**Fig. V.7.c**). Similarly, these variants were subcultured on blood agar plates, then phages ES17 or HP3's respective EOP were assessed again on each of them, according to the phage they had previously been exposed to. As opposed to *P. aeruginosa*, though, only one variant (1/5) displayed a mildly reduced EOP, the majority (4/5) displaying no modification in phage susceptibility. Long-read sequencing of these variants did not highlight any genomic modification in line with described BPR-acquisition pathways, only highlighting one deletion causing a non-synonymous frameshift variation in a hypothetical protein in one ES17-induced variant (**Table V.2**).

Similar to *P. aeruginosa* assays, OmniLog growth assays in similar conditions yielded significantly lower rates of bacterial "escape" growth over the same timeframe compared to GOACs, this time for both phages and either at MOI 1 (ES17: $p = 0.0014$; HP3: $p = 0.0034$) or at MOI 10 (ES17: $p = 0.0061$; HP3: $p = 0.0242$) (**Fig. V.7.d-e**).

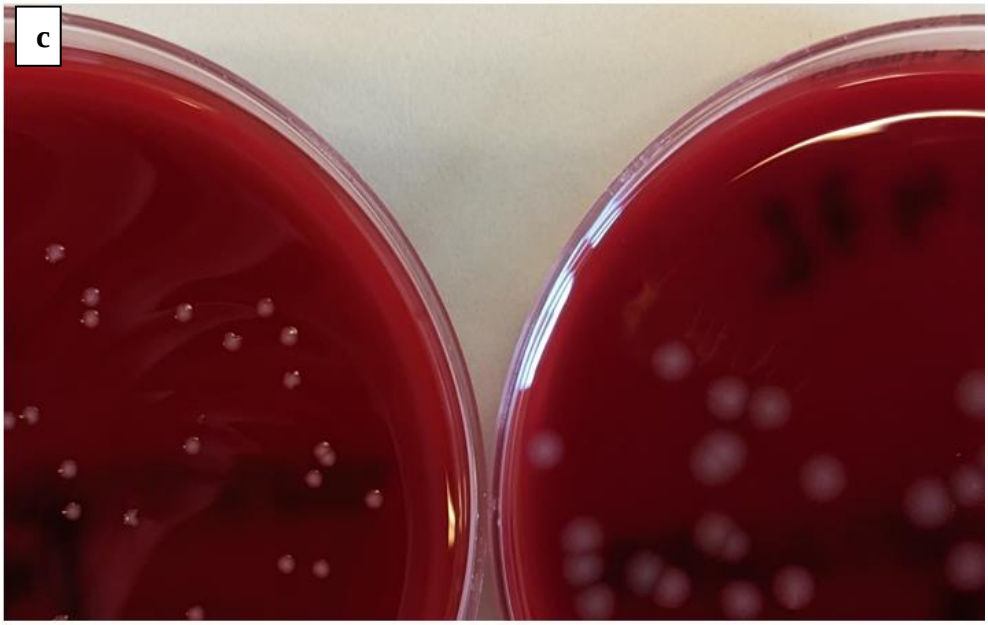
a


E. coli + Dual phage (ES17 + HP3)
MOI1 - sequential (Δ1h)
Difference to initial bacterial load (log10 CFU/mL)
3,0
2,0
1,0
,0
-1,0
-2,0
0
1
2
3
Days
Conditions
Ec - 1h - Dual phage MOI1
Ec - 1h - ES17 MOI1
Ec - 1h - HP3 MOI1
Ec only

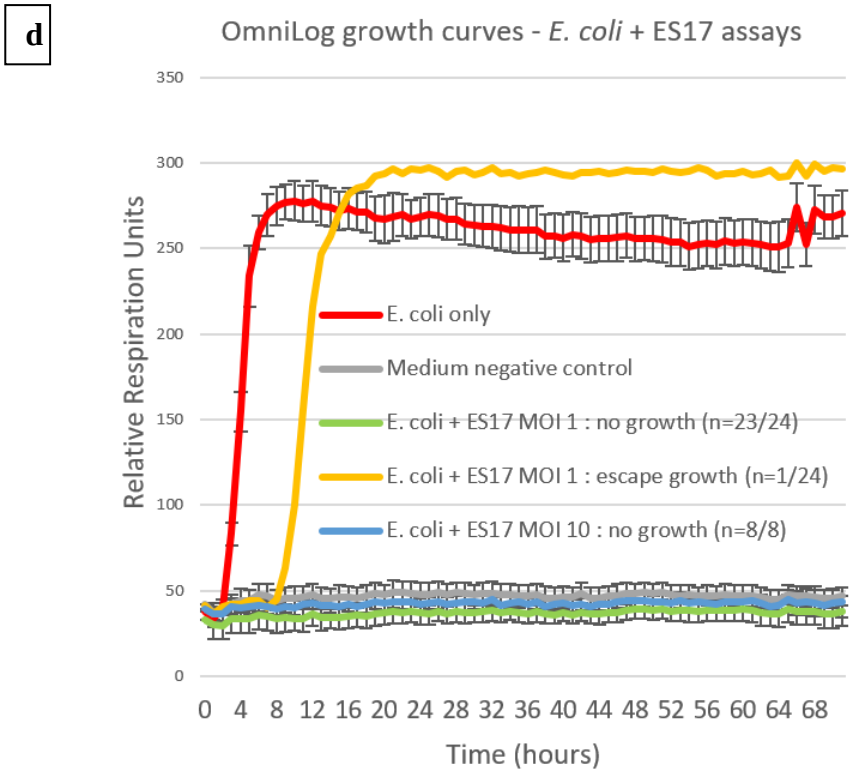
b


E. coli + Dual phage (ES17 + HP3)
MOI10 - sequential (Δ1h)
Difference to initial bacterial load (log10 CFU/mL)
3,0
2,0
1,0
,0
-1,0
-2,0
0
1
2
3
Days
Conditions
Ec - 1h - Dual phage MOI10
Ec - 1h - ES17 MOI10
Ec - 1h - HP3 MOI10
Ec only

4587


c

4588


d
OmniLog growth curves - E. coli + ES17 assays
Relative Respiration Units
350
300
250
200
150
100
50
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68
Time (hours)
E. coli only
Medium negative control
E. coli + ES17 MOI 1 : no growth (n=23/24)
E. coli + ES17 MOI 1 : escape growth (n=1/24)
E. coli + ES17 MOI 10 : no growth (n=8/8)

e

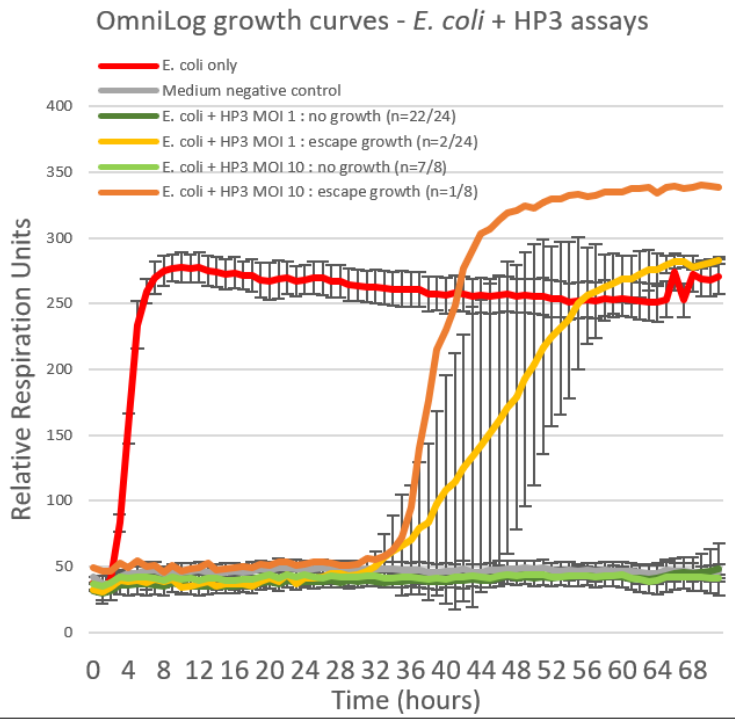


Fig. V.7 | *E. coli* ST131: dual phage and OmniLog assays. *E. coli* titers evolution when introduced in bicellular GOACs with a 50%/50% combination of phage ES17 and phage HP3 in comparison with both mono-phage counterparts, at MOI = 1 (n=3 each) **(a)** or MOI = 10 (n=3 each) **(b)**. Example of modified *E. coli* ST131 colony morphology (left, variant ST131-V1 see Table V2) after exposition to HP3 at MOI = 1 compared to wild type colony morphology (right) **(c)**. *E. coli* with either ES17 **(d)** or HP3 **(e)** control growth curves in static *in vitro* conditions using an OmniLog automated incubator, bacterial respiration units being measured as a proxy for bacterial growth (initial bacterial load 10^5 CFU/well ; n = 24 wells for each MOI = 1 assay, 8 wells for each MOI = 10 assay, 16 wells for *E. coli* only and 16 wells for negative control ; error bars represent +/-1 standard deviation of the mean). *[Ec: E. coli ; MOI: Multiplicity Of Infection]*

4602

Isolate	Colony phenotype	Inductor phage	Inductor phage's EOP	Inductor phage's susceptibility	Mutations and/or structural variations
ST131-V1	Bright white dwarf colonies	HP3	0,116	Partly susceptible	No detectable non-synonymous variation
ST131-V2	Bright white colonies	ES17	1	Fully susceptible	No detectable non-synonymous variation
ST131-V3	Hazy gray large colonies	ES17	1	Fully susceptible	Deletion causing a frameshift variant in a hypothetical protein
ST131-V4	Bright grey colonies	HP3	1	Fully susceptible	No detectable non-synonymous variation
ST131-V5	Bright white colonies	ES17	1	Fully susceptible	No detectable non-synonymous variation

253

Table V.2 | *E. coli* variants typing. Summary of phage susceptibility testing and genomic characterization of 5 variants of *E. coli* ST131 retrieved after co-evolution with phage ES17 or HP3 in GOACs.

V.4.5 Serum-supplemented OmniLog assays

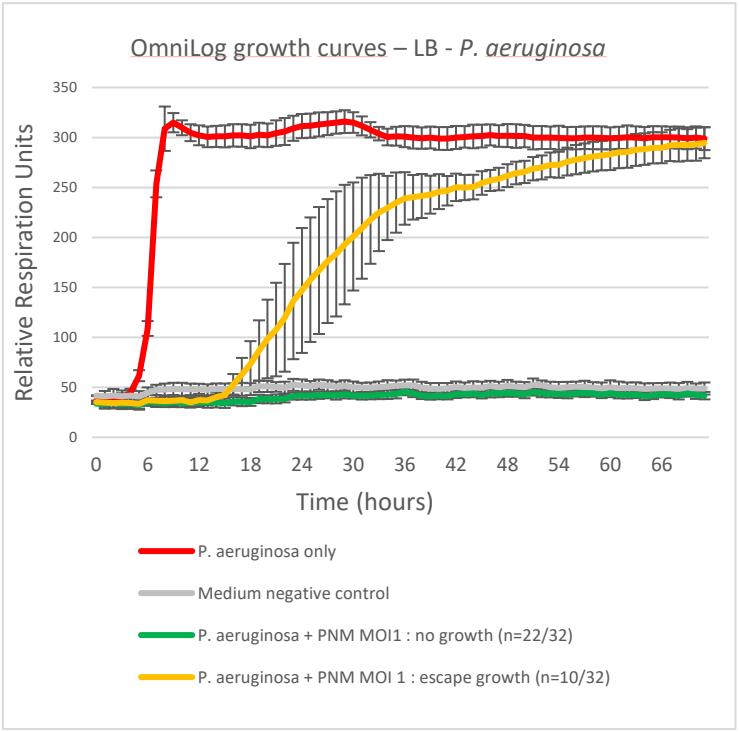
To maximize comparability, above-mentioned "control" OmniLog assays were eventually replicated with the modification of a single parameter: supplementing the LB culture medium with 20% FBS, to reach identical FBS proportion as in the medium infused in GOACs.

Comparison between these three pairs of assays (i.e. *P. aeruginosa* CN573 and PNM, *E. coli* ST131 and ES17, *E. coli* ST131 and HP3) is represented in Figure 8.

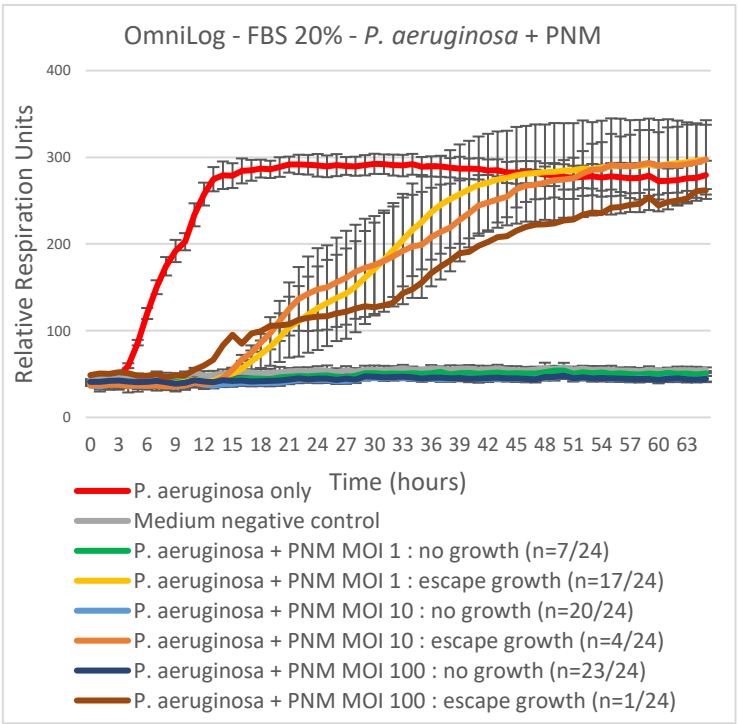
Qualitatively, FBS supplementation does not seem to significantly modify the escape growth profile, still starting at least six hours after the bacteria-only positive control but eventually reaching a similar plateau by the end of the 72h period.

Quantitatively, on the other hand, FBS supplementation leads to a significantly higher rate of bacterial escape growth than in its FBS-free counterpart when considering the *P. aeruginosa*-PNM duet (MOI 1 : $p = 0.0064$), but not in *E. coli*-ES17 (MOI 1 : $p = 1$; MOI 10 : $p = 1$) nor in *E. coli*-HP3 (MOI 1 : $p = 1$; MOI 10 : $p = 0.443$) assays. As opposed to standard FBS-free OmniLog assays, FBS-supplemented OmniLog outcomes for *P. aeruginosa*-PNM (MOI 1) in terms of escape growth rate thus do not differ significantly from that of GOACs ($p = 0.545$). FBS-supplementation's deteriorating effect on PNM outcomes at MOI 1 in these OmniLog assays can however be surpassed by the use of higher MOIs like 10 and 100 (**Fig. V.8**).

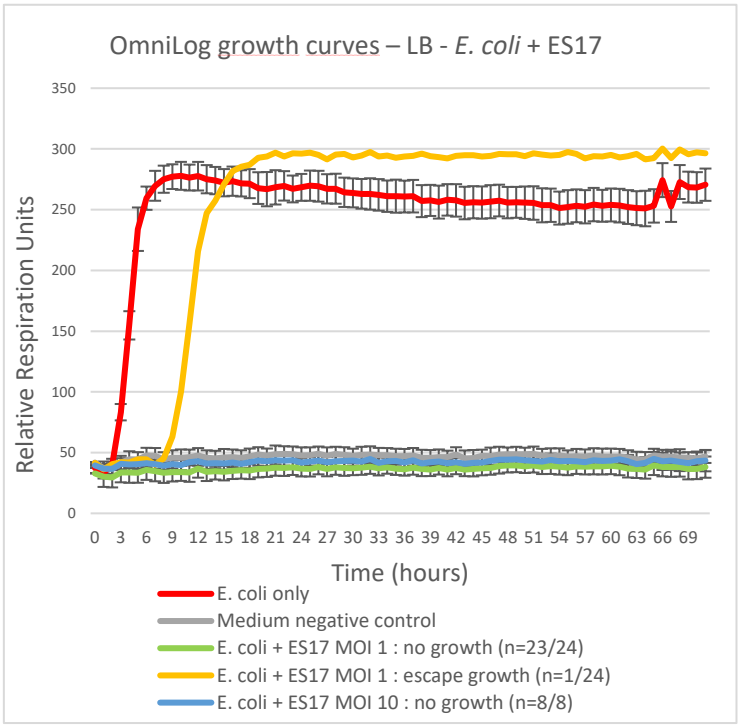
a


OmniLog growth curves – LB - P. aeruginosa
Relative Respiration Units
350
300
250
200
150
100
50
0
0 6 12 18 24 30 36 42 48 54 60 66
Time (hours)
P. aeruginosa only
Medium negative control
P. aeruginosa + PNM MOI1 : no growth (n=22/32)
P. aeruginosa + PNM MOI 1 : escape growth (n=10/32)

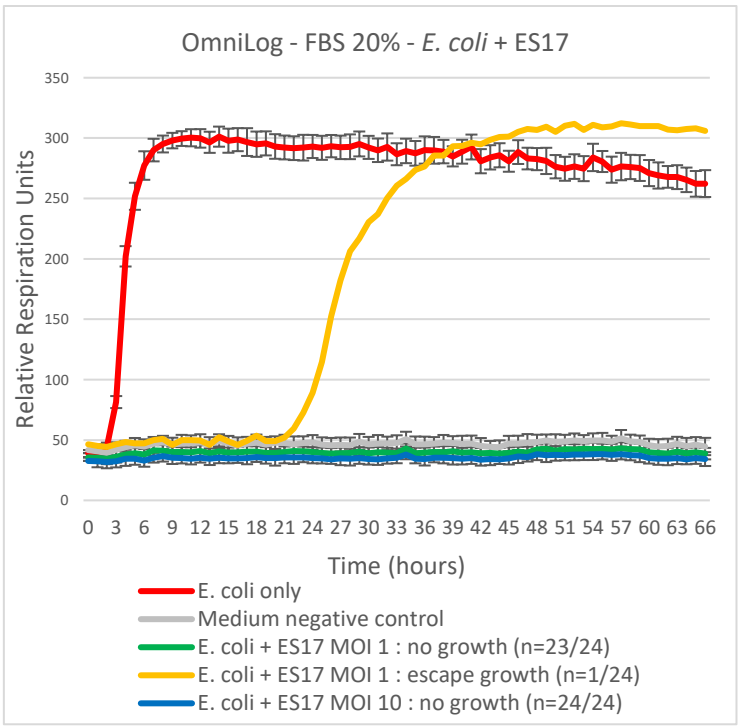
b


OmniLog - FBS 20% - P. aeruginosa + PNM
Relative Respiration Units
400
300
200
100
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63
Time (hours)
P. aeruginosa only
Medium negative control
P. aeruginosa + PNM MOI 1 : no growth (n=7/24)
P. aeruginosa + PNM MOI 1 : escape growth (n=17/24)
P. aeruginosa + PNM MOI 10 : no growth (n=20/24)
P. aeruginosa + PNM MOI 10 : escape growth (n=4/24)
P. aeruginosa + PNM MOI 100 : no growth (n=23/24)
P. aeruginosa + PNM MOI 100 : escape growth (n=1/24)

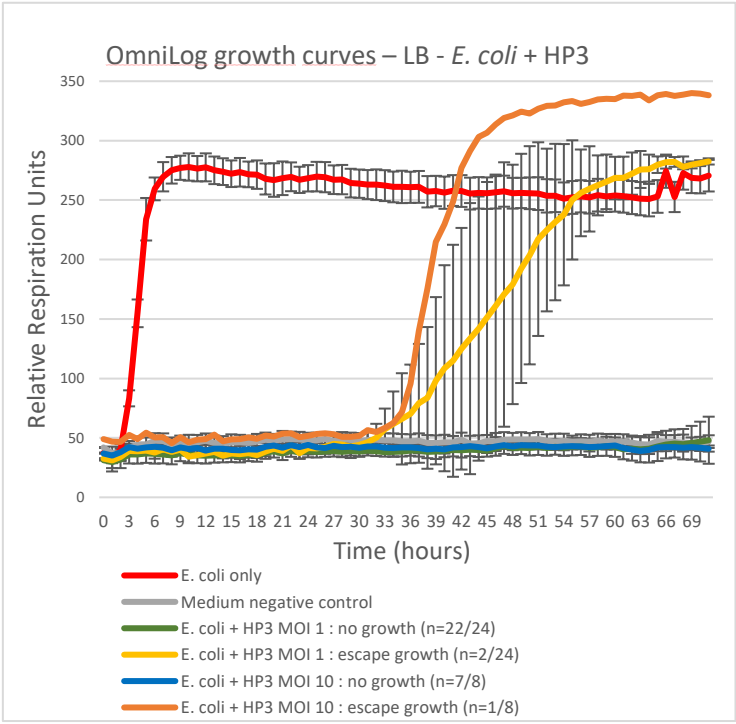
c


OmniLog growth curves – LB - E. coli + ES17
Relative Respiration Units
350
300
250
200
150
100
50
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63 66 69
Time (hours)
E. coli only
Medium negative control
E. coli + ES17 MOI 1 : no growth (n=23/24)
E. coli + ES17 MOI 1 : escape growth (n=1/24)
E. coli + ES17 MOI 10 : no growth (n=8/8)

d


OmniLog - FBS 20% - E. coli + ES17
Relative Respiration Units
350
300
250
200
150
100
50
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63 66
Time (hours)
E. coli only
Medium negative control
E. coli + ES17 MOI 1 : no growth (n=23/24)
E. coli + ES17 MOI 1 : escape growth (n=1/24)
E. coli + ES17 MOI 10 : no growth (n=24/24)

e


OmniLog growth curves – LB - E. coli + HP3
Relative Respiration Units
350
300
250
200
150
100
50
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63 66 69
Time (hours)
E. coli only
Medium negative control
E. coli + HP3 MOI 1 : no growth (n=22/24)
E. coli + HP3 MOI 1 : escape growth (n=2/24)
E. coli + HP3 MOI 10 : no growth (n=7/8)
E. coli + HP3 MOI 10 : escape growth (n=1/8)

f

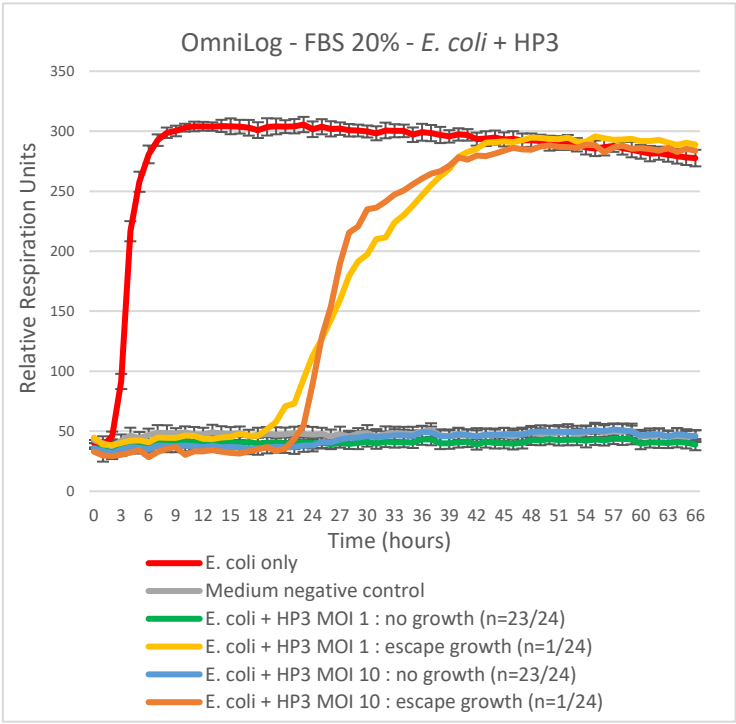

OmniLog - FBS 20% - E. coli + HP3
Relative Respiration Units
350
300
250
200
150
100
50
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63 66
Time (hours)
E. coli only
Medium negative control
E. coli + HP3 MOI 1 : no growth (n=23/24)
E. coli + HP3 MOI 1 : escape growth (n=1/24)
E. coli + HP3 MOI 10 : no growth (n=23/24)
E. coli + HP3 MOI 10 : escape growth (n=1/24)

Fig. V.8 | Serum-supplemented OmniLog assays. Replicating previous OmniLog assays with the supplementation of 20% fetal bovine serum (the same proportion as in the GOAC culture medium) to LB yields to significantly higher bacterial escape growth rates in *P. aeruginosa* – PNM assays **(a, b)**, but not in either *E. coli* – ES17 **(c, d)** nor in *E. coli* – HP3 **(e, f)** assays.

V.5 Discussion

This work investigated bacterial-phage dynamics in GOACs under numerous culture conditions, focusing on two bacterial species: *P. aeruginosa* and *E. coli*. The choice to focus on these two is based on experimental but also clinical relevance, taking inspiration from our clinical practice in *Cliniques universitaires Saint-Luc* ([CUSL] - Brussels, Belgium). In CUSL, one of the most important subsets of patients concerned by MDR bacteria asymptomatic carriage, and potential associated infections, are pediatric liver transplant recipients.

On the one hand, *P. aeruginosa*, while not the most frequently encountered carriage in the CUSL subpopulation, has led on occasion to devastating carriage-infection conversions, responsible for high morbidity and limited therapeutic options[266]. Furthermore, its carriage rate appears as one of the most frequent ones in another large comparable series of pediatric liver transplant recipients in Korea, advocating for a variable clinical relevance according to local epidemiology[206]. On the other hand, choosing to also work with *E. coli* appeared locally relevant given its high carriage

incidence in the CUSL subpopulation. Moreover, this choice gave us access to a pair of corresponding anti-*E. coli* phages whose respective properties had recently been extensively described, and in particular their MBPs or lack thereof[241]. This made the *E. coli* bacteria-phage duet an ideal candidate to transition this clinically-inspired problematic to a digestive research model such as the GOAC.

Green and colleagues' findings from comparative oral administration of two phages harboring MBPs (phage ES17) or not (phage HP3) led them to conclude that the former was more efficient at lysing *E. coli* ST131 in the digestive tract of mice. They also showed that this difference was likely due to the inhibitory (or at least bacteria-protective) effect exerted by mucins on phages devoid of MBPs, as opposed to their enhancing effect on a phage harboring MBPs. In this case, the MBPs of phage ES17 seem mediated by a tail fiber protein specifically binding to heparan sulfate proteoglycans, ubiquitous glycoproteins present at the basement and surface membranes of cells. Their findings are consistent with a previous paradigm proposed by Barr and colleagues, the "Bacteriophage Adherence to Mucus" (BAM) model[270]. The BAM paradigm implies that some phages constitutive of animal commensal microbiomes can reach a state of prosperity in parietal mucus-rich interfaces by harboring MBPs. In the case of BAM, these MBPs have been described as mediated by several subtypes of immunoglobulin-like domains[270, 271, 272, 273, 274]. This prosperity allows for more likely phage-bacteria encounters in these mucus-rich interfaces, inducing bactericidal properties constitutive of a "non-host-derived immunity", for example able to prevent bacterial translocation from the gut lumen to the bloodstream[270, 272].

Except for the fact that MBPs of ES17 are not mediated by immunoglobulin-like domains, both of these groups' conclusions coincide.

To investigate whether these conclusions could be extrapolated to GOACs, the GOACs required a proper parietal mucus secretion, since it is an essential condition to the BAM paradigm. Histological sections of Alcian blue-stained inlet catheters of GOACs have suggested that sustained cell culture in our GOACs could induce the production of a thin continuous apical mucus layer. Furthermore, mucin quantification by specific quantitative ELISA tests illustrated that our GOACs produced detectable quantities of targeted mucins MUC5AC and MUC2, and that the production of MUC2 was increased in bicellular GOACs compared to both mono-cellular counterparts. We had initially chosen to supplement 100% Caco-2 GOACs with LS 174T cells because of LS 174T's well documented MUC2 secretion potential, whereas transcriptomic studies had suggested Caco-2 cells had no significant MUC2 production in two-dimensional static culture[275]. Surprisingly, 100% Caco-2 GOACs did not produce significantly less MUC2 than 100% LS 174T GOACs. Besides different outcome measurement (mRNA vs antigen), this difference might be induced by the different culture conditions (two-dimensional static vs three-dimensional dynamic), in line with previous reports of MUC2 secretion by Caco-2 cells in GOACs[276]. Optimal MUC2 secretion, achieved in bicellular GOACs, seemed primordial given its indispensable role in organizing the architecture of the mucus-embedded microbiome along the gut walls[277]. For this reason, bicellular GOACs were considered the most physiologically relevant after the initial comparative series, and were the only type of GOACs used in *E. coli* assays. MUC2 shapes the parietal mucus layer into two contrasted sublayers, an inner dense

sublayer hostile to microbiological life, lining the gut epithelium's apical surface and protecting it from direct bacterial contact. On the other hand, the outer sublayer is partly disaggregated, and its looser structure fosters thriving microbiological life. In *P. aeruginosa* assays, the relative lack of MUC2 abundance in 100% Caco-2 GOACs might explain why they did not seem as protective to bacterial prosperity as the 50%/50% Caco-2 – LS 174T bicellular GOACs, under the same phage conditions.

In *P. aeruginosa* assays, bacterial "escape" growth following initial decline under phage pressure was correlated with the emergence of various *P. aeruginosa* colonies of modified morphology. We postulated that these morphological modifications were likely correlated with fitness advantages allowing for bacterial growth under phage pressure, such as the acquisition of BPR mechanisms. This seemed especially likely given the absence of morphologically modified colonies in bacteria-only control GOACs. Consistent with this hypothesis, PNM susceptibility testing performed by DAO assay on these variants always illustrated a dramatic loss in phage-susceptibility, consistent with clinical thresholds used to qualify BPR (EOP < 0.01). Accordingly, sequencing of these variants revealed systematic acquisition of T4P-related genomic modification, known as causative of BPR phenotypes[140, 266]. Other genomic modifications were also detected, some of which also known to cause BPR, affecting structures and functions such as LPS biosynthesis, pyomelanin metabolism conferring the brown coloration of some variants (*hmgA*), or O-antigen-related virulence (*galU*)[278]. These alterations highlight a key limitation of GOACs since all of their associated fitness costs could be comparatively significantly higher in human hosts. First, possible loss of virulence due to T4P, LPS or *galU*-related alterations

could hinder *in vivo* bacterial survival according to the PIVT paradigm[266, 278]. Second, potential alterations in the T6SS could hinder *in vivo* and especially digestive fitness of *P. aeruginosa* given the key role played by T6SS in inter-species bacterial competition in a given ecological niche, a feature that is likely not valued in a GOAC devoid of any bacterial competition[279]. It should be noted that the synonymous variant found in this work and suspected to be part of the T6SS complex does not necessarily result in an absence of phenotypic modification regarding T6SS structure or function since codon usage bias has been shown to potentially generate phenotypic changes between synonymous codons, including in *P. aeruginosa*[280, 281]. Lastly, pyomelanogenic phage-resistant *P. aeruginosa* variants related to *hmgA* alterations are correlated to higher susceptibility to colistin, a last resort antibiotic against *P. aeruginosa*, suggesting possibly synergistic properties of phage-antibiotic interactions on these variants[278, 282].

In *E. coli* assays, a similar approach of phenotypic variant analysis during bacterial "escape" growth did not provide similar results. As opposed to the *P. aeruginosa* assays, ES17 and HP3 susceptibility testing performed by DAO assays on these variants never showed dramatic decreases in EOP, suggesting an absence of BPR acquisition in these variants. These results could suggest gut-specific mechanisms of *E. coli* phage-resistance or phage-evasion, that cannot be reproduced in phage-bacteria assays outside of the GOAC and thus can possibly not be correlated to expected BPR-inducing genomic alterations in these variants. This hypothesis goes in line with previous findings by Chibani-Chennoufi and colleagues[283]. Their findings illustrate that *E. coli* existing as long-term colonizers in the gut of mice are significantly more resistant to digestive phage-therapy than *E. coli* newly

introduced in the gut, while both *E. coli* display similar phage-susceptibility outside of the gut environment. Whether these gut-specific determinants of phage-resistance were even mediated by bacteria themselves was not certain, suggesting possible mechanisms of "non-bacterial phage resistance". Consistent with this paradigm, sequencing did not highlight any genomic modification in line with described BPR-acquisition pathways, only highlighting one deletion causing a non-synonymous frameshift variation in a hypothetical protein in one ES17-induced variant. This suggests that in-GOAC *E. coli* adaptation towards phage-evasion might be based on dynamic gene regulation or even on non-bacterial determinants rather than on durable genomic alterations. This hypothesis goes in line with recent in-depth investigation by Lourenço and colleagues, illustrating the tripartite (bacteria, phage, gut environment) nature of this significant *in vitro – in vivo* mismatch, highlighting the importance of dynamic gene regulation in gut-colonizing *E. coli* and its lower associated fitness costs compared to stable genomic alterations like loss-of-function mutations[284]. The same group had previously illustrated that the gut's architectural features themselves played a key role in creating "bacterial refuges" in the mucosal environment, creating conditions for long-term bacteria-phage prosperity at the spatial level, regardless of the respective development of BPR and associated phage co-evolution[285]. The potential of phages to evolve along gut-specific mutational pathways has also been recently documented, including in GOAC models[249].

P. aeruginosa and *E. coli* assays also differ by their comparative outcomes in OmniLog assays, especially when considering serum-supplemented assays. Besides carrying serum-free OmniLog assays as "neutral" *in vitro* comparators, we decided to replicate them using a similar

FBS-proportion as in the GOAC infusion medium so as to rule out the contribution of serum itself in the GOAC-OmniLog outcomes' differences. The rationale behind this is the previous description of phage-deteriorating properties of serum, mostly reported in *Staphylococcus aureus*, though varying vastly among *S. aureus* strains[286]. These properties appear to be likely correlated to IgGs, thought to compete with phages for specific surface receptors, hampering phage adsorption[286, 287]. Works reporting similar mechanisms in *P. aeruginosa* and *E. coli* phages are rarer, tend to illustrate these properties in phage-induced immunized serum and not in supposedly "naïve" serum such as FBS in our case, and report potentially high variance between phages and between replicates[288, 289, 290]. Here, we illustrated significantly different serum-induced effects on OmniLog outcomes according to species : apparently neutral for *E. coli* and both ES17 and HP3 phages, but significantly phage-deteriorating for *P. aeruginosa* and PNM, although this appears fully reversible by the use of higher MOIs. This represents an additional layer of analytical complexity in what fosters differences between OmniLog and GOAC outcomes, but also between GOAC and mice outcomes, as will be discussed below.

In both *P. aeruginosa* and *E. coli* assays, sequential introduction of bacteria followed by phages after a 1h static incubation led to more bacteria-favoring outcomes than simultaneous introduction of the same quantities of bacteria and phages. In sequential introduction conditions, as opposed to some simultaneous conditions, no GOAC ever reached undetectable bacterial titers at any timepoint, regardless of phages used and corresponding MOI. This bacteria-favoring effect of bacterial chronological pre-existence in a digestive PT model is again consistent with the aforementioned findings of

Chibani-Chennoufi and colleagues[283]. This might look discouraging given that bacterial pre-existence is by definition present in the clinical problematic initially tackled in this work: phage-mediated digestive decolonization. However, opposite condition of phage pre-existence in the GOACs did not lead to more encouraging outcomes either, phage pre-treatment of the GOACs proving unable to prevent ulterior bacterial colonization of the GOACs, as opposed to previous findings from comparable assays[271].

Our *E. coli* GOAC assays generated results that seem contradictory to some of Green and colleagues' findings after similar assays in mice. Indeed, as opposed to their results, MBPs of phage ES17 could not revert the bacteria-favoring scenario of our *E. coli*-infected GOACs compared to phage HP3, devoid of MBPs. ES17 did not perform better at lowering GOAC bacterial titers than HP3 in any but one culture condition. Indeed, a slightly better effect at 24h was observed during continuous phage infusion condition, achieving significantly lower bacterial titer than bacteria-only control GOACs but not significantly lower than HP3 GOACs. This is highly contrasting with the spectacular bactericidal efficiency of ES17 in Green and colleagues' mice model, especially compared to the inefficiency of HP3. Several factors might explain these differences. First, GOACs used in this work do not replicate the complex aero-anaerobic gradients found it the live digestive tract, though previous complex iterations of GOACs have been able to achieve it[247]. For example, as a facultative aero-anaerobe, *E. coli* has been shown to endure dynamic gene regulation during gut colonization to handle the aero-anaerobic switch, in parallel to the development of phage-resistance mechanisms when phages were co-introduced in the gut[284]. Anaerobic conditions have been suspected to influence phage-bacteria interactions, including adsorption rates

and frequency of BPR-inducing mutations[291]. Second, it has been increasingly documented that phages, while incapable of actively infecting eukaryotic cells, can however get internalized in them. This is notably the case in the gut where phages in the digestive lumen are subject to an epithelial uptake by transcytosis, representing a potential sink for phages and limiting their in-gut availability[292]. It is unknown whether the intensity of this uptake-mediated loss in available phages is similar in GOACs and mice. Third, and in relation to the aforementioned digestive phage transcytosis mechanisms, GOACs lack any immune system. While increasing evidence is advocating for significant interactions between gut resident phages and the mucosal immune system, notably explained via their transcytosis potential in both health and disease, little is known about the acute implication of the immune system on newly introduced phages in the gut community in the scenario of oral PT[293, 294]. The idea that phage-immune interactions could result in increased bactericidal properties is supported by previous findings[295]. Fourth, tissue architecture in GOACs probably only resumes part of the complexity of that of the live digestive tract: the aforementioned spatial determinants influencing gut-specific phage-bacteria coexistence are thus likely different too[285]. Lastly, and perhaps most importantly, the GOACs used in this work lack any sort of intra- and inter-specific bacterial competition, as the only bacterial strain present in a GOAC is the one directly targeted by the co-introduced phages. This is a critical limitation, as these GOACs will likely not reproduce the synergistic bactericidal action mediated by PT's lytic pressure and the relative fitness costs associated to the development of bacterial "escape" growth under this pressure in a competitive microbiological environment. This thus fails to reproduce one example in the

larger family of "vice effects" that are highly suspected to influence PT's outcomes[266]. In line with this hypothesis, recent findings by Forsyth and colleagues have illustrated that even though fitness costs associated with BPR itself in digestive *E. coli* ST131 were not intrinsically high, significant synergistic bactericidal effect against *E. coli* ST131 could be achieved by combining PT and the addition of a probiotic *E. coli* Nissle, leading to a dramatic increase in BPR-associated fitness costs[296]. It should also be noted that, either in Green and colleagues' work or in ours, comparing ES17 with HP3 does not allow for proper evaluation of the net, isolated properties of ES17's MBPs. This could only be achieved by comparing wild type ES17 with an engineered loss-of-function mutant of this phage, similarly to what others have investigated with anti-*E. coli* phage T4 and its MBP-inducing outer capsid protein Hoc[271].

Besides aforementioned factors potentially explaining GOAC-mice mismatch, there are a number of other limitations to our work. GOACs are work-intensive models. Developing and validating them in a reproductible fashion starting from no previous experience will often require a lot of troubleshooting. While the user can vastly increase the model's complexity and physiological relevance by implementing a baseline simplified healthy gut microbiota and working with double-channel GOACs, anaerobic culture chambers, peristaltic pumps, oxygen sensors, heat-inactivating chips, self-dispensing output pumps or other custom features, all of these additional aspects will increase the tedious nature of GOAC development and handling [247, 249]. This limitation also restricts numerical reproducibility: a single user working with a single multichannel pump can only handle series of a certain number of GOACs at a time, and the delay needed to bring them from liquid

PDMS to ready-to-infect GOACs creates a certain time inertia. For this reason, our will to test numerous conditions in a limited timeframe led us to work only with triplicates, which sometimes showed suboptimal internal reproducibility. This was for example the case with simultaneous phage-bacteria introduction conditions, which had already been shown to generate considerable stochastic variations, each GOAC representing a unique, idiosyncratic ecosystem[249]. Another limitation to this work is the main focus on bacterial outcomes at the expense of phage outcomes. Phages were not quantified in egressing GOAC medium to establish in-GOAC evolution of the PFU/mL phage titers in parallel to bacterial titers ; likewise, co-evolved phages were not retrieved for comparative genomic analysis. An example of such parallel monitoring has been reported using quantitative polymerase chain reaction (qPCR), in a way that can be automated[249]. Such automation can also allow significantly finer time granularity compared to our once-a-day monitoring. On the other hand, plating-based CFU monitoring allowed us to screen, isolate and type phage- and/or GOAC-induced bacterial variants in a way qPCR could not have achieved if used alone. These approaches might be seen as complementary, but could also be complemented or in some cases replaced by shotgun metagenomics.

V.6 Conclusion

In conclusion, we investigated phage-bacteria dynamics in GOACs, first with *P. aeruginosa* and corresponding phage PNM, then with *E. coli* and corresponding phages ES17 and HP3. These assays were modulated around numerous conditions of phage titers, phage MBPs, phage-bacteria timing of introduction, and epithelial cell lines. GOACs were shown to achieve relevant mucin secretion, with an optimized secretion of MUC2 in bicellular GOACs, which were mostly used in this work. Though the aforementioned varied growth conditions generated distinct results, our assays have drawn a general trend of bacterial prosperity and resilience in these models, showing a significantly higher rate of "escape" growth under phage lytic pressure than in more neutral static *in vitro* assays. Comparative assays illustrated that serum-induced loss of phage activity might explain this mismatch for *P. aeruginosa*, but likely not for *E. coli*, at least considering the three phages we worked with. Other factors increasing bacterial advantage were use of bicellular GOACs and temporal pre-existence of the bacteria in the model compared to phages. In *E. coli* assays, temporal pre-existence of phages or continuous infusion of phages in the model could not shift this balance of power. Increasing MOI of single phages or using a two-phages combination led to temporarily lower bacterial titers, though never reaching the threshold of bacterial undetectability at any timepoint. Phenotypic and genomic characterization of bacterial variants thriving under phage pressure suggests that achieving phage-evasion can either be linked to durable genomic modifications causing BPR, or to suspected dynamic gene regulation and non-bacterial phage-evasion determinants. The contribution of these

respective mechanisms might greatly vary between bacterial species, as might serum-related loss of phage activity. The occurrence of this suspected dynamic gene regulation was not directly confirmed in our model, highlighting the need for transcriptomic or proteomic monitoring in future GOAC-mediated phage research, for example. Importantly, in *E. coli* assays, this gut-specific bacterial prosperity could not be reverted either by the use of a phage harboring MBPs (ES17) compared to an MBP-lacking phage (HP3), potentially contradicting recent findings from similar assays in mice model. The numerous differences between GOACs and mice models might explain these differences. Various perspectives pave the way for future phage research in GOACs or other digestive models, like the critical need for intra- and inter-species bacterial competition, the investigation of a "block and replace" synergistic therapy using a phage-probiotic combination, the study of BPR- and non-BPR-associated fitness costs, the extrapolation of phage-antibiotic interactions in these models, and the comparative genomic analysis between phage-escaping bacterial variants from GOACs compared to those from control static *in vitro* conditions.

Chapter VI: General discussion and concluding remarks

VI.1 Main achievements of the thesis

Both the fragilities and strengths of our doctoral research work might reside in the fact that it consisted of clearly distinct sub-works differing in topic, aim, methodology, or analytical material – from the almost purely clinical to the fundamental. However, our aim was to convince the reader that these subparts were all articulated around a central topic, phage therapy (PT), acting as a common denominator. Another axis of understanding can also be the chronological imbrication of all subparts based on a clinical "*princeps* case" from which almost all subsequent questions derived, well in the tradition of reverse translational ("bedside to bench") research.

This *princeps* case is the one presented in Section III.1, telling the complex story of the PT performed in a one-year-old toddler suffering from post-liver transplantation (LT) XDR-*P. aeruginosa* infection. To summarize this chronological imbrication, this case made us :

- First, capitalize on this initial, burgeoning clinical experience to treat other well-selected cases with PT, and characterize several clinically-related features that we believed influenced the successful or disappointing therapeutic outcome.

- Second, in a preventive approach, to investigate the "carriage-infection conversion" paradigm that likely led to the life-threatening infection of the *princeps* case, with:

- An observational part of a clinical study (OPTICS – *Oral Phage Therapy against Intestinal Carriage of Superbugs*) that aimed to better understand the microbiological characteristics of the pediatric LT population in our clinical practice (*Cliniques universitaires Saint-Luc – CUSL).*

- An interventional part of the same clinical study that theoretically allowed us to treat some of these patients by oral PT in the pre-LT period in order to selectively decolonize a previously detected asymptomatic digestive carriage of an MDR/XDR/PDR bacteria.

- Lastly, the use of a modern in-flow three-dimensional cell culture model, the "Gut-On-A-Chip" (GOAC) to investigate bacterial dynamics under phage lytic pressure inside a pseudo-digestive mucus-rich environment, in order to shed light on what could modulate or hinder oral PT's efficacy in the aforementioned OPTICS application.

We will now briefly summarize the findings of all of them.

Our clinical experience included the organization, administration, supervision and analysis of seven PT cases. All of these cases resisted conventional SOC therapy including antibiotic treatment (including several sequential regimens) and other adapted medico-surgical measures. These seven cases covered, as mentioned, a diversity of clinical indications, routes of administration, and targeted pathogens (Sections III.2 & III.3 – **Tables III.2 & III.3**). PT safety assessment according to clinical and basic biological

follow-up (including hemogram, C-reactive protein, renal function and liver enzymes repeatedly before, during and after PT) was reassuring in all of them. PT was always performed in a way we thought respected a few logical "success criteria" recently proposed by Stacey and colleagues for the discrimination of efficacy outcome in PT clinical trials, which can be summed up as "the right amount of the right phages, delivered at the right place, at the right time during infection progression, to target a sufficient number of bacterial cells". Yet, two out of these seven cases were not followed by favorable microbiological nor clinical outcomes, suggesting these criteria might fit a "necessary but insufficient" scenario. Criteria for PT failure in the first of those two cases have been discussed in a specific publication and are likely mostly related to the partial phage coverage of a multi-bacterial infection. Explanation of PT failure in the second case, a chronic *P. aeruginosa* osteomyelitis, remains more elusive, and is probably linked to the lack of initial intra-operative phage lavage.

The other five cases were followed on the short term by durable microbiological clearance of the targeted pathogens in the treated site(s) and by either partly or fully favorable clinical progression. This timely correlation in cases previously refractory to SOC therapy suggests a causal nature between PT application and both microbiological and clinical improvement. However, PT on its own might not be responsible in full for this. Indeed, antibiotic therapy, even when considered unhelpful when used alone in these cases, was still pursued in parallel to PT in all seven cases. Two examples of phage-antibiotic interactions investigation based on clinical cases were presented, and showed *in vitro* synergistic properties for some antibiotics in both cases. However, *in vitro* phage-rifampin antagonistic interaction at

specific phage-antibiotic titer combinations was also suspected on MSSA, consistent with other previously published *in vitro* results. Lastly, some of our phage-antibiotic interactions results seemed to contradict general trends recently proposed in literature, such as the tendency of anti-ribosomal antibiotics to lead to phage-antibiotic antagonism: this was not observed in the two cases in which we tested such interactions in vitro, namely with gentamycin in case 2, nor clindamycin in case 1[297, 298]. The reasons underlying these discrepancies are still obscure and deserve utmost attention in future research given their potential implications on clinical decisions. We suspect they might depend, for example, on *in vitro* methodology and material, and on tested bacterial strain.

Other complex mechanisms at the intersection between phages, antibiotics, human host and targeted bacteria have also been studied in some of these cases. Phage Immune Neutralization (PIN) was illustrated in two cases receiving intravenous PT. Its development seemed to be phage- and host immune system-dependent, and was not correlated to pejorative microbiological or clinical outcomes of PT. Bacterial phage resistance (BPR) was especially studied in the *princeps* case, its genomic basis being more thoroughly characterized (regarding *P. aeruginosa*'s resistance to phage PNM) by comparative chromosomic sequencing of various phage-resistant and phage-susceptible isolates. Again, the development of such BPR was not correlated to PT failure. This apparent paradox made us question the incurrence of possible phage-induced virulence tradeoffs (PIVT). These were investigated in *Galleria mellonella* larvae and HeLa cells. However, we could not illustrate a specific correlation pattern between phage-resistant phenotype and loss of virulence in this specific case.

The OPTICS (*Oral Phage Therapy against Intestinal Carriage of Superbugs*) study was then set up to try to better understand and possibly prevent such "carriage-infection conversion" mechanisms that led to the devastating post-LT infection in the *princeps* case. Indeed, digestive carriers of some MDR bacterial species are generally described to be at higher risk of subsequently developing an actual infection due to the same bacterial species, this "conversion" being typically facilitated by a number of risk factors such as heavy surgical procedures or intensive care unit stay. We wanted to address this problematic in the specific case of such conversions in pediatric LT recipients – a specifically large patient population in our local clinical practice (Cliniques universitaires Saint-Luc, Brussels, Belgium).

The OPTICS study screened 41 potential LT recipients and 33 LT procedures in 32 of them (one re-transplantation). Epidemiological findings derived from this study have to be interpreted cautiously given its small sample size. Yet, it notably included the absence of correlation between pre-LT carriage of a shortlist of MDR bacteria (mostly ESBL-Enterobacterales), and post-LT risk of early bacterial infection, contrarily to what has been generally reported in other series. We suspected this might be due to a specificity of our clinical setting acting as an interfering variable: the systematic use of temocillin in surgical antibiotic prophylaxis (SAP) regimen in these patients, used for its efficacious spectrum on aforementioned ESBL-Enterobacterales. When considering only bacterial carriages that resisted temocillin, this statistically significant carriage-to-infection correlation was restored, and though the subpopulation of "temocillin-resistant carriers" represented only 15% of patients enduring LT, it accounted for 50% of its incurred infections in our series. These findings warrant confirmation by

pursuing such protocol, but suggests the need to re-discuss the potential for SAP personalization in these patients.

Personalizing the pre-LT management of MDR bacteria carriage and mitigation of associated infectious risk might not, however, rely solely on antibiotic use. As previous works have pointed out, the use of targeted oral PT in this specific digestive decolonization indication could yield interesting potential, and cause less collateral harm to the rest of the gut microbiota than the use of oral broad-spectrum antibiotics, for example. We experimentally modeled the basics of this scenario by using a special microfluidic three-dimensional tubular cell culture model: the Gut-On-A-Chip (GOAC). These GOACs aimed at mimicking a series of basic characteristics of the digestive tract for bacteria and phages to be implemented into. GOACs were cultured under a large number of experimental conditions, varying in cell type, bacterial load, timing and duration of phage administration. Two bacterial species of interest were investigated in the GOACs: first *P. aeruginosa* and corresponding phage PNM, then *E. coli* and corresponding phages ES17 and HP3. Our assays have drawn a general trend of bacterial prosperity and resilience in GOACs, showing a significantly higher rate of "escape" growth under phage lytic pressure than in more neutral static *in vitro* assays performed using the OmniLog® automated incubation system. Factors increasing this bacterial advantage were use of bicellular GOACs, which showed optimized MUC2 mucin secretion, and temporal pre-existence of the bacteria in the model compared to phages. In *E. coli* assays, this gut-specific bacterial prosperity could not be reverted by the use of phage ES17, a phage harboring mucus-binding properties (MBP), compared to an MBP-lacking phage (HP3): this tends to contradict the findings of recent works using the

same phages in similar indications in mice. Phenotypic and genomic characterization of bacterial variants thriving under phage pressure suggests that achieving phage-evasion can sometimes be linked to durable genomic modifications causing BPR, but not necessarily, leading us to suspect evasion pathways relying on dynamic gene regulation that we did not directly observe. The respective contributions of these mechanisms might greatly vary between bacterial species.

VI.2 Limitations

Numerous limitations to our work can be underlined: they already have been in the respective "Discussion" sections of the different chapters of this work. We will summarize their main points.

The description of our clinical-based work has included the reporting of clinical and microbiological outcomes in a handful of PT cases covering a variety of clinical indications, targeted bacterial species and degrees of therapeutic emergency, ranging from hyper-acute to hyper-chronic. This mini-series should of course be considered as an encouraging start, but in its current state, the combination of its scarcity and its diversity on all of these levels makes it near-impossible to draw any robust conclusion from it. The green flags / red flags and do's/don'ts recommendations we formulated at the end of Chapter III should thus be considered cautiously, and are admittedly the product of clinical and empirical intuition and common sense rather than set in stone dogmas.

Perhaps the greatest limitation to the progression of clinical phage research to the next level of "evidence-generating research", such as RCTs for example, lies in regulatory constraints. Indeed, as already mentioned in Section II.2.2, the aforementioned clinical cases have been given access to PT thanks to a specific Belgian regulation exploiting the single paradigm in which medicinal products can access a drug status without requiring market authorization: the magistral preparation. In our cases, and provided a handful of aforementioned conditions were respected, performing PT in these distinct individual patients was relatively easy and could theoretically be considered SOC. However, this magistral preparation paradigm only allows the administration of PT in what could be called a "repeatedly punctual" way, in other words in a tailored case-by-case fashion and not in the systematic and reproductible way of a prospective clinical trial protocol. This is due to the fact that this "magistral phage" regulation is surpassed by other binding constraints in the case of clinical trials, the most limiting being the obligation to comply to Good Manufacturing Practices (GMP), which is not officially the case of the phages available to us at the moment. In our view, this represents a very puzzling paradox, at least at the Belgian scale: on the one hand, administration of PT in individual, specific, non-research-related patients is flexible and could progressively become mundane in the coming years, but on the other hand, carrying the much needed clinical trials to validate specific indications and administration protocols is an extremely daunting task. In most other fields of so-called "experimental medicine", the opposite would seem far more logical. This perplexing situation can only continue to fuel the skepticism that some medical professionals still hold against PT. This situation needs to be addressed urgently.

Limitations related to the OPTICS trial have been addressed in Section IV.4. They mostly focus on the impossibility to include any of the screened patients into the interventional protocol *per se*, which aimed to administer oral PT in the pre-LT period in order to attempt selective digestive decolonization of MDR bacteria. The reasons besides this included organizational constraints that should progressively be overcome with our growing experience. But the most important factor in this relative failure lies in the suboptimal bactericidal spectrum of the available anti-*E. coli* and anti-*K. pneumoniae* phage panels that were available at the time. The mechanisms underlying this insufficient coverage are very different between these two species. The relatively small number of available anti-*E. coli* phages at the moment is likely mostly caused by low demand. Conversely, the challenge faced when constituting an anti-*K. pneumoniae* "broad-spectrum phage panel" is mostly of technical, microbiological nature, strict capsule-type-dependency of most anti-*K. pneumoniae* phages lying at the center of the problematic.

Finally, limitations of our work with the GOAC model are especially numerous, and have been discussed in Section V.5. In summary, the key limitations first include some regarding GOAC-mice reproducibility of results, especially when comparing the differential effect of MBPs on anti-*E. coli* bactericidal effect. Reasons explaining this mismatch might include absence of aero-anaerobic parietal-luminal gradient, absence of mucosal immune system, and perhaps most importantly absence of intra- and inter-specific bacterial competition. Other key limitations derive from more technical constraints: inability to self-build a dual-channel GOAC with a pseudo-gut-vascular interface in our given material, logistical and

chronological constraints, or inability to obtain a pure MBP-knockout phage comparator in anti-*E. coli* assays, for example. Lastly, the absence of a paralleled monitoring of in-model phage titers and, ideally, of acquired phage genetic modifications is an important missing link: further research should ideally systematically include these, in order to study not only phage-derived bactericidal effect but rather phage-bacteria co-evolution.

All these limitations indeed foster enthusiasm for future, more in-depth phage research in organ-on-chips models; we will also then be reminded to keep a critical eye when comparing such assays' results with those obtained from animals or humans.

VI.3 Future perspectives

The rebound in interest for PT-related research is relatively recent. This leaves room for legitimate enthusiasm in conquering the countless uncharted territories that this field of research still contains, both on the more clinical and more fundamental ends of the spectrum. Based on our experience and personal intuition and interest, we will only mention a handful of them here.

We will start with what we think are key perspectives for future phage *therapy* research, i.e. phage research with a clinical orientation. These can be summarized as a deepening of our initial investigations on specific mechanisms lying at the numerous intersections between the four "players" of PT: the phage, the bacterium it targets, the human host it is administered

to, and the antibiotics that are almost always administered conjointly (**Fig. VI.1**).

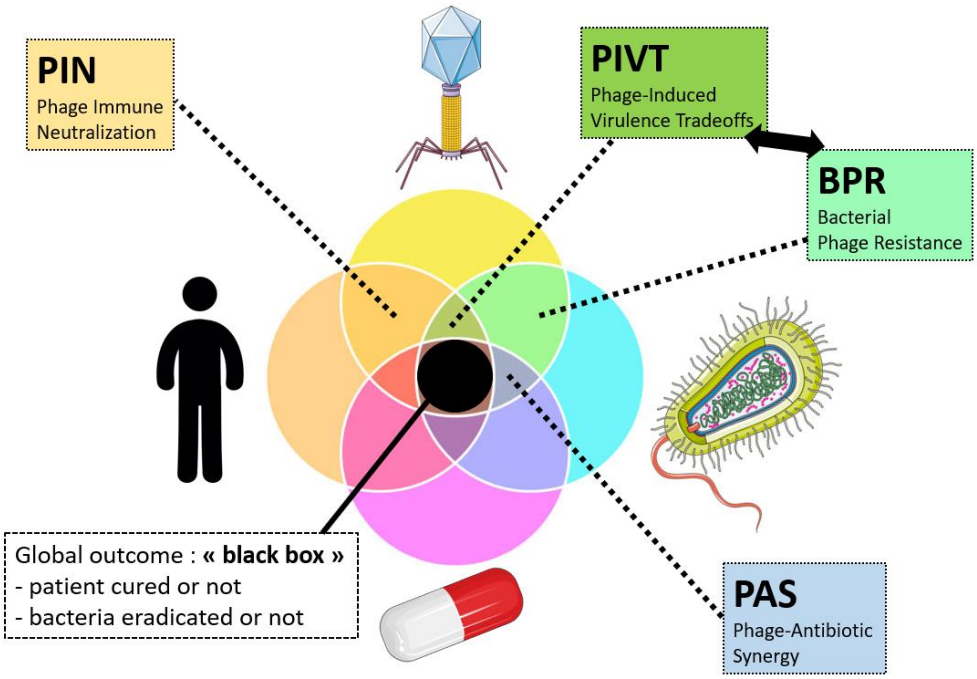


Fig. VI.1 | The four-player scenario of phage therapy. Complex concepts lie at the respective intersections between these four entities, obviously not limited to the four listed here. Listing and investigating them further will likely allow for a finer understanding of the central intersection which can be seen as the "gross result" of PT, i.e. the direct outcome on patient clinical improvement or lack thereof, and/or targeted bacterium eradication or persistence. *From open-access graphical items only.*

First, the clinical implications of phage immune neutralization (PIN) must be better understood. As previously mentioned, development of PIN during the primary exposition to a given phage likely does not mitigate the

efficacy outcomes of this first episode of PT, but sporadic literature has suggested that it might however mitigate its efficacy upon re-exposition[128]. In the eyes of some, this might not look like a major issue: if the number of patients treated by PT worldwide is already small, the number of those that will require several distinct episodes of PT using the same phage(s) is even significantly smaller. Yet, in a perspective where PT could become increasingly used in the next decades, this small number could grow steadily. Thus, deciphering whether PIN is a major concern especially for repeated PT seems crucial. In particular, it seems important to better understand the determinants of potential immune cross-reactivity (between the exposed phage and other phages, but also between the exposed phage and a non-phage antigen) and the factors influencing the immunogenicity of a given phage antigen. To experimentally investigate whether prophylactic immunosuppressive medications could reliably prevent PIN from happening would also be interesting, since part of our clinical work suggests PIN is probably impaired in immunocompromised patients.

Second, clarifying the consequences and management of bacterial phage resistance (BPR), and its potentially associated phage-induced virulence tradeoffs (PIVT), still remains an exciting challenge. Paradoxical mismatches between development of BPR and favorable clinical and microbiological evolution during PT have been reported in one of our aforementioned clinical cases, and our attempts at explaining this, notably through the PIVT paradigm, have not been fully conclusive. Knowing whether PIVT is or is not the main mechanism by which BPR is not necessarily correlated to PT failure seems crucial, yet researchers focusing on this precise topic are very scarce at the moment. As a consequence,

methodological recommendations on how to best investigate the development of PIVT are still missing, the diverging results from our *G. mellonella* and our HeLa cells virulence assays being illustrative of this. Knowing whether PIVT is mostly related to dynamic *in vivo* gene regulation or to durable genomic alterations for example would deeply modify our experimental approaches. Similarly, knowing whether PIVT can be intentionally favored by the use of (a combination of) specific phages targeting specific bacterial receptors could also greatly orientate ulterior efforts in the constitution of clinically-relevant phage panels.

Third, phage-antibiotic interactions (PAI) and among them phage-antibiotic synergies (PAS) also warrant further clinically-applied research. Often misperceived by clinicians as a potential *replacement* for antibiotics, phages might in turn rather prove to be their greatest allies. In several of our aforementioned clinical cases, *in vitro* results were favorable to potential PAS mechanisms in most cases, derived from 96-well microplates filled with various combinations of phages and antibiotics at diverse titers and analyzed in an automated bacterial incubator (OmniLog). Yet, in other cases, antagonistic properties at very specific respective titers were also present. Assessing the best methodology, both in material but also in statistical analysis, to conclude to *in vitro* PAS are urgently needed: so far, in practice, this has mostly been evaluated simply on the intuitive visual analysis of the OmniLog curves. In the case of OmniLog, systematically measuring objective curve metrics such as area under the curve (AUC) and time to reach $AUC_{50\%}$, for example, could be interesting. To add to the complexity, and perhaps most importantly, little to nothing is known about the *in vitro* to *in vivo* translatability of these results. However, on that level, a soon-to-be-

published review of the last 100 cases of personalized PT cases in Belgium included, as one of its key findings, the significantly higher therapeutic efficacy of phage-antibiotic combination therapy versus PT alone[299]. This fosters enthusiasm for ulterior research in *in vivo* PAS.

Lastly, our preliminary work in GOACs also suggests numerous paths for improvement. Digestive applications of phages might be the most complex and least understood of all so far, because besides targeting a given bacterial species, they are also destined to mix and interfere with the healthy gut microbiota in both its bacterial and phage compartments. Still, this does not even consider the additional interactions of these phages with structural determinants such as pH, shear stress or, as was investigated in GOACs, mucus. Determining the ideal experimental model to best represent this complexity in full is a challenge, each technical compromise risking over-simplification as a consequence, and potentially associated mismatches between models, as we have experienced. Still, recent research works have triggered renewed enthusiasm in digestive phage research. Federici and colleagues have published an impressive set of results suggesting encouraging efficacy of a pre-adapted oral anti-*K. pneumoniae* PT in the treatment of *K. pneumoniae*-mediated inflammatory bowed disease in mice[300]. Fujiki and Schnabl recently published a consequent body of work reviewing in detail the results of previous works regarding digestive PT, and proposed an important conceptual basis for the treatment of liver diseases by specifically targeting bacterial strains incriminated in the development and progression of non-alcoholic fatty liver disease (like high-alcohol-producing *K. pneumoniae* for example) or alcoholic liver disease (like cytolytic *E. faecalis* for example)[301].

VI.4 Conclusion

Antimicrobial resistance and more specifically bacterial resistance to antibiotics is a global threat for human health, and its burden is only expected to steadily increase in the coming decades. Complementary therapeutic tools are thus urgently needed in the fight against infections caused drug-resistant bacteria. Among them, phage therapy, the therapeutic use of lytic bacteriophage viruses, is likely the one to generate the most interest in recent clinical and fundamental research. Our application of phage therapy in seven patients to treat very diverse clinical and bacteriological indications confirms its promising potential. However, pursuing the description of factors influencing therapeutic success or failure seems crucial, and we have proposed a series of personal experience-based suggestions on that regard. Complex mechanisms lying at different intersections between phages, antibiotics, bacteria and human patients might shed light on these factors, such as the occurrence of phage immune neutralization, bacterial phage resistance, or phage-antibiotic interactions.

We also planned to use oral phage therapy as a way to specifically eradicate a series of digestive carriage of drug-resistant bacterial strains in children awaiting liver transplantation. We postulated that this could mitigate the early infectious risk after liver transplantation, which remains a major cause of morbidity and mortality in these patients. Observational findings from over forty screened patients and over thirty liver transplantation performed among them suggest that personalization of surgical antibiotic prophylaxis in accordance to previously identified drug-resistant carriage might be an interesting way to mitigate this risk. The scenario of oral phage

therapy in this decolonization indication was implemented in an *in vitro* "Gut-On-A-Chip" model. The results derived from it suggest a multi-factorial gut-specific bacterial prosperity compared to more neutral *in vitro* conditions, a resilience to phage bactericidal effect that could not be reverted by the mucus-binding properties harbored by specific phages in our model.

Phages' Future – ideas for tomorrow's researchers

Making antibiotics great again: molecular basis, predictive models and technological standardization for Phage-Antibiotic Interactions

Phage-Antibiotic Interactions: pharmacodynamics and pharmacokinetics in the experimental and clinical assessment of in vitro to in vivo extrapolation

Phage Immune Neutralization: experimental characterization of phage- and host-mediated contributing factors and clinical implications

Phage(s) therapy: from competition to synergy, characterization of the receptor- and metabolism-related factors of inter-phage interactions

Bacterial Phage Resistance: integrating phages, receptors and bacterial metabolic pathways in predictive approaches of genomic alterations and dynamic regulations

The vice phenomenon: structural and molecular relevance of experimental models in phage-induced tradeoffs to consolidate therapeutic conclusions

Non-canonical phage therapy: key fields, assets and hurdles for phage-related therapeutics beyond the treatment of bacterial infections

References

1. Antibiotic resistance threats in the United States, 2019. (2019).

2. Mohapatra SS, Dwibedy SK, Padhy I. Polymyxins, the last-resort antibiotics: Mode of action, resistance emergence, and potential solutions. *J Biosci* **46**, (2021).

3. Magiorakos AP, *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* **18**, 268-281 (2012).

4. Davies J, Davies D. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* **74**, 417-433 (2010).

5. Rosini R, Nicchi S, Pizza M, Rappuoli R. Vaccines Against Antimicrobial Resistance. *Frontiers in Immunology* **11**, (2020).

6. OCDE. *Stemming the Superbug Tide* (2018).

7. Aiello AE, Larson EL, Sedlak R. Hidden heroes of the health revolution Sanitation and personal hygiene. *American Journal of Infection Control* **36**, S128-S151 (2008).

8. Hutchings MI, Truman AW, Wilkinson B. Antibiotics: past, present and future. *Current Opinion in Microbiology* **51**, 72-80 (2019).

9. Plackett B. Why big pharma has abandoned antibiotics. *Nature* **586**, S50-S52 (2020).

10. Abraham EP, Chain E. An enzyme from bacteria able to destroy penicillin. 1940. *Rev Infect Dis* **10**, 677-678 (1988).

11. Klein S, *et al.* Rapid Development of Cefiderocol Resistance in Carbapenem-resistant Enterobacter cloacae During Therapy Is Associated With Heterogeneous Mutations in the Catecholate Siderophore Receptor cirA. *Clinical Infectious Diseases* **74**, 905-908 (2021).

12. Hota B, *et al.* Predictors of Clinical Virulence in Community-Onset Methicillin-Resistant Staphylococcus aureus Infections: The Importance of USA300 and Pneumonia. *Clinical Infectious Diseases* **53**, 757-765 (2011).

13. Remschmidt C, Schröder C, Behnke M, Gastmeier P, Geffers C, Kramer T. Continuous increase of vancomycin resistance in enterococci causing nosocomial infections in Germany − 10 years of surveillance. *Antimicrobial Resistance & Infection Control* **7**, (2018).

14. Willmann M, Marschal M, Hölzl F, Schröppel K, Autenrieth I, Peter S. Time Series Analysis as a Tool To Predict the Impact of Antimicrobial Restriction in Antibiotic Stewardship Programs Using the Example of Multidrug-Resistant Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy* **57**, (2013).

15. Baker SJ, Payne DJ, Rappuoli R, De Gregorio E. Technologies to address antimicrobial resistance. *Proceedings of the National Academy of Sciences* **115**, 12887-12895 (2018).

16. Naughton B. *The Chinese economy : transitions and growth / Barry Naughton*. Cambridge, Mass. : MIT Press, c2007. (2007).

17. Su J-Q, *et al.* Metagenomics of urban sewage identifies an extensively shared antibiotic resistome in China. *Microbiome* **5**, 84 (2017).

18. Rahman S, Hollis A. The effect of antibiotic usage on resistance in humans and food-producing animals: a longitudinal, One Health analysis using European data. *Front Public Health* **11**, 1170426 (2023).

19. Tan SY, Khan RA, Khalid KE, Chong CW, Bakhtiar A. Correlation between antibiotic consumption and the occurrence of multidrug-resistant organisms

in a Malaysian tertiary hospital: a 3-year observational study. *Sci Rep* **12**, 3106 (2022).

20. Chatterjee A, *et al.* Quantifying drivers of antibiotic resistance in humans: a systematic review. *The Lancet Infectious Diseases* **18**, e368-e378 (2018).

21. Larsson DGJ, Flach C-F. Antibiotic resistance in the environment. *Nat Rev Microbiol* **20**, 257-269 (2022).

22. McEwen SA, Collignon PJ. Antimicrobial Resistance: a One Health Perspective. In: *Antimicrobial Resistance in Bacteria from Livestock and Companion Animals*) (2018).

23. Browne AJ, *et al.* Global antibiotic consumption and usage in humans, 2000–18: a spatial modelling study. *The Lancet Planetary Health* **5**, e893-e904 (2021).

24. Pouwels KB, *et al.* Association between use of different antibiotics and trimethoprim resistance: going beyond the obvious crude association. *J Antimicrob Chemother* **73**, 1700-1707 (2018).

25. Murray CJL, *et al.* Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet* **399**, 629-655 (2022).

26. Collignon P, Beggs JJ, Walsh TR, Gandra S, Laxminarayan R. Anthropological and socioeconomic factors contributing to global antimicrobial resistance: a univariate and multivariable analysis. *The Lancet Planetary Health* **2**, e398-e405 (2018).

27. Li W, *et al.* Association between antibiotic resistance and increasing ambient temperature in China: an ecological study with nationwide panel data. *The Lancet Regional Health – Western Pacific* **30**, (2023).

28. Hay SI, *et al.* Measuring and mapping the global burden of antimicrobial resistance. *BMC Med* **16**, 78 (2018).

29. Allel K, *et al.* Global antimicrobial-resistance drivers: an ecological country-level study at the human–animal interface. *The Lancet Planetary Health* **7**, e291-e303 (2023).

30. Global status report on road safety.). World Health Organization (2018).

31. O'Neill J. Review on Antimicrobial Resistance Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations.) (2014).

32. de Kraker MEA, Stewardson AJ, Harbarth S. Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050? *PLOS Medicine* **13**, e1002184 (2016).

33. Poudel AN, *et al.* The economic burden of antibiotic resistance: A systematic review and meta-analysis. *PLoS One* **18**, e0285170 (2023).

34. Patel J, *et al.* Measuring the global response to antimicrobial resistance, 2020–21: a systematic governance analysis of 114 countries. *The Lancet Infectious Diseases* **23**, 706-718 (2023).

35. Wang X, Yu D, Chen L. Antimicrobial resistance and mechanisms of epigenetic regulation. *Front Cell Infect Microbiol* **13**, (2023).

36. Reygaert WC. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol* **4**, 482-501 (2018).

37. Weigel LM, Steward CD, Tenover FC. gyrA mutations associated with fluoroquinolone resistance in eight species of Enterobacteriaceae. *Antimicrob Agents Chemother* **42**, 2661-2667 (1998).

38. Lorusso AB, Carrara JA, Barroso CDN, Tuon FF, Faoro H. Role of Efflux Pumps on Antimicrobial Resistance in Pseudomonas aeruginosa. *Int J Mol Sci* **23**, (2022).

39. Ocampo-Sosa AA, *et al.* Alterations of OprD in carbapenem-intermediate and -susceptible strains of Pseudomonas aeruginosa isolated from patients with bacteremia in a Spanish multicenter study. *Antimicrob Agents Chemother* **56**, 1703-1713 (2012).

40. Aljeldah MM. Antimicrobial Resistance and Its Spread Is a Global Threat. *Antibiotics* **11**, 1082 (2022).

41. Darby EM, *et al.* Molecular mechanisms of antibiotic resistance revisited. *Nat Rev Microbiol* **21**, 280-295 (2023).

42. Bhatnagar K, Wong A. The mutational landscape of quinolone resistance in Escherichia coli. *PLOS ONE* **14**, e0224650 (2019).

43. Rodríguez-Beltrán J, DelaFuente J, León-Sampedro R, MacLean RC, San Millán Á. Beyond horizontal gene transfer: the role of plasmids in bacterial evolution. *Nat Rev Microbiol* **19**, 347-359 (2021).

44. Kent AG, Vill AC, Shi Q, Satlin MJ, Brito IL. Widespread transfer of mobile antibiotic resistance genes within individual gut microbiomes revealed through bacterial Hi-C. *Nature Communications* **11**, 4379 (2020).

45. von Wintersdorff CJH, *et al.* Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in Microbiology* **7**, (2016).

46. Li B, Qiu Y, Song Y, Lin H, Yin H. Dissecting horizontal and vertical gene transfer of antibiotic resistance plasmid in bacterial community using microfluidics. *Environ Int* **131**, 105007 (2019).

47. Chen J, *et al.* Genome hypermobility by lateral transduction. *Science* **362**, 207-212 (2018).

48. Humphrey S, *et al.* Bacterial chromosomal mobility via lateral transduction exceeds that of classical mobile genetic elements. *Nat Commun* **12**, 6509 (2021).

49. Erdmann S, Tschitschko B, Zhong L, Raftery MJ, Cavicchioli R. A plasmid from an Antarctic haloarchaeon uses specialized membrane vesicles to disseminate and infect plasmid-free cells. *Nat Microbiol* **2**, 1446-1455 (2017).

50. Abe K, Nomura N, Suzuki S. Biofilms: hot spots of horizontal gene transfer (HGT) in aquatic environments, with a focus on a new HGT mechanism. *FEMS Microbiol Ecol* **96**, (2020).

51. Wright GD. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat Rev Microbiol* **5**, 175-186 (2007).

52. D'Costa VM, McGrann KM, Hughes DW, Wright GD. Sampling the antibiotic resistome. *Science* **311**, 374-377 (2006).

53. D'Costa VM, *et al.* Antibiotic resistance is ancient. *Nature* **477**, 457-461 (2011).

54. Rice LB. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infect Dis* **197**, 1079-1081 (2008).

55. Rice LB. Progress and challenges in implementing the research on ESKAPE pathogens. *Infect Control Hosp Epidemiol* **31 Suppl 1**, S7-10 (2010).

56. World Health O. Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis.). World Health Organization (2017).

57. O'Neill J. *Tackling drug-resistant infections globally: final report and recommendations*. Government of the United Kingdom (2016).

58. Mölstad S, *et al.* Lessons learnt during 20 years of the Swedish strategic programme against antibiotic resistance. *Bull World Health Organ* **95**, 764-773 (2017).

59. Terreni M, Taccani M, Pregnolato M. New Antibiotics for Multidrug-Resistant Bacterial Strains: Latest Research Developments and Future Perspectives. *Molecules* **26**, 2671 (2021).

60. 2021 antibacterial agents in clinical and preclinical development: an overview and analysis.). World Health Organization (2022).

61. Patel TS, *et al.* Defining access without excess: expanding appropriate use of antibiotics targeting multidrug-resistant organisms. *Lancet Microbe*, (2023).

62. Rolain JM, Abat C, Jimeno MT, Fournier PE, Raoult D. Do we need new antibiotics? *Clinical Microbiology and Infection* **22**, 408-415 (2016).

63. Rello J, Bunsow E, Perez A. What if there were no new antibiotics? A look at alternatives. *Expert Review of Clinical Pharmacology* **9**, 1547-1555 (2016).

64. Livermore DM, *et al.* Discovery research: the scientific challenge of finding new antibiotics. *Journal of Antimicrobial Chemotherapy* **66**, 1941-1944 (2011).

65. Ghosh C, Sarkar P, Issa R, Haldar J. Alternatives to Conventional Antibiotics in the Era of Antimicrobial Resistance. *Trends in Microbiology* **27**, 323-338 (2019).

66. Browne K, *et al.* A New Era of Antibiotics: The Clinical Potential of Antimicrobial Peptides. *Int J Mol Sci* **21**, 7047 (2020).

67. Kollef MH, Betthauser KD. Monoclonal antibodies as antibacterial therapies: thinking outside of the box. *The Lancet Infectious Diseases* **21**, 1201-1202 (2021).

68. Simonis A, *et al.* Discovery of highly neutralizing human antibodies targeting Pseudomonas aeruginosa. *Cell* **186**, 5098-5113.e5019 (2023).

69. Maura D, Ballok AE, Rahme LG. Considerations and caveats in anti-virulence drug development. *Current Opinion in Microbiology* **33**, 41-46 (2016).

70. Defoirdt T. Quorum-Sensing Systems as Targets for Antivirulence Therapy. *Trends in Microbiology* **26**, 313-328 (2018).

71. Cavallo FM, Jordana L, Friedrich AW, Glasner C, van Dijl JM. Bdellovibrio bacteriovorus: a potential 'living antibiotic' to control bacterial pathogens. *Crit Rev Microbiol* **47**, 630-646 (2021).

72. Twort FW. AN INVESTIGATION ON THE NATURE OF ULTRA-MICROSCOPIC VIRUSES. *The Lancet* **186**, 1241-1243 (1915).

73. d'Herelle M. Sur un microbe invisible antagoniste des bacilles dysentériques. *Acta Kravsi*, (1961).

74. Abedon ST, Thomas-Abedon C, Thomas A, Mazure H. Bacteriophage prehistory. *Bacteriophage* **1**, 174-178 (2011).

75. Cisek AA, Dąbrowska I, Gregorczyk KP, Wyżewski Z. Phage Therapy in Bacterial Infections Treatment: One Hundred Years After the Discovery of Bacteriophages. *Curr Microbiol* **74**, 277-283 (2017).

76. Ackermann HW. The first phage electron micrographs. *Bacteriophage* **1**, 225-227 (2011).

77. Summers WC. Bacteriophage Therapy. *Annu Rev Microbiol* **55**, 437-451 (2001).

78. Chanishvili N. Phage therapy--history from Twort and d'Herelle through Soviet experience to current approaches. *Adv Virus Res* **83**, 3-40 (2012).

79. Pirnay J-P, *et al.* Introducing yesterday's phage therapy in today's medicine. *Future Virology* **7**, 379-390 (2012).

80. Thiel K. Old dogma, new tricks--21st Century phage therapy. *Nat Biotechnol* **22**, 31-36 (2004).

81. Dedrick RM, *et al.* Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant Mycobacterium abscessus. *Nat Med* **25**, 730-733 (2019).

82. Gill JJ, Hyman P. Phage Choice, Isolation, and Preparation for Phage Therapy. *Current Pharmaceutical Biotechnology* **11**, 2-14 (2010).

83. Chan BK, Abedon ST, Loc-Carrillo C. Phage cocktails and the future of phage therapy. *Future Microbiol* **8**, 769-783 (2013).

84. Pirnay JP, *et al.* The phage therapy paradigm: prêt-à-porter or sur-mesure? *Pharm Res* **28**, 934-937 (2011).

85. Yang Q, Le S, Zhu T, Wu N. Regulations of phage therapy across the world. *Front Microbiol* **14**, 1250848 (2023).

86. Fauconnier A. Regulating phage therapy: The biological master file concept could help to overcome regulatory challenge of personalized medicines. *EMBO Rep* **18**, 198-200 (2017).

87. Willy C, *et al.* Phage Therapy in Germany—Update 2023. *Viruses* **15**, 588 (2023).

88. Fauconnier A. Phage Therapy Regulation: From Night to Dawn. *Viruses* **11**, (2019).

89. Fauconnier A. Guidelines for Bacteriophage Product Certification. *Methods Mol Biol* **1693**, 253-268 (2018).

90. Pirnay J-P, Verbeken G. Magistral Phage Preparations: Is This the Model for Everyone? *Clinical Infectious Diseases* **77**, S360-S369 (2023).

91. Pirnay JP, *et al.* The Magistral Phage. *Viruses* **10**, 64-70 (2018).

92. Association WM. World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. *JAMA* **310**, 2191-2194 (2013).

93. Regulation (EU) No 536/2014 of the European Parliament and of the Council of 16 April 2014 on clinical trials on medicinal products for human use, and repealing Directive 2001/20/EC Text with EEA relevance. (ed Council EPa). Official Journal of the European Union L158/1 (2014).

94. Keen EC. A century of phage research: bacteriophages and the shaping of modern biology. **37**, 6-9 (2015).

95. Ackermann HW. Phage classification and characterization. *Methods Mol Biol* **501**, 127-140 (2009).

96. Turner D, Kropinski AM, Adriaenssens EM. A Roadmap for Genome-Based Phage Taxonomy. *Viruses* **13**, 506 (2021).

97. Leprince A, Mahillon J. Phage Adsorption to Gram-Positive Bacteria. *Viruses* **15**, (2023).

98. Campbell A. The future of bacteriophage biology. *Nature Reviews Genetics* **4**, 471-477 (2003).

99. Dennehy JJ, Abedon ST. Phage infection and lysis. *Bacteriophages: biology, technology, therapy*, 341-383 (2021).

100. Riede I, Degen M, Henning U. The receptor specificity of bacteriophages can be determined by a tail fiber modifying protein. *Embo j* **4**, 2343-2346 (1985).

101. Bertozzi Silva J, Storms Z, Sauvageau D. Host receptors for bacteriophage adsorption. *FEMS Microbiol Lett* **363**, (2016).

102. Enikeeva FN, Severinov KV, Gelfand MS. Restriction-modification systems and bacteriophage invasion: who wins? *J Theor Biol* **266**, 550-559 (2010).

103. Shen Y, Loessner MJ. Beyond antibacterials – exploring bacteriophages as antivirulence agents. *Current Opinion in Biotechnology* **68**, 166-173 (2021).

104. Hobbs Z, Abedon ST. Diversity of phage infection types and associated terminology: the problem with 'Lytic or lysogenic'. *FEMS Microbiol Lett* **363**, (2016).

105. Silpe JE, Bassler BL. A Host-Produced Quorum-Sensing Autoinducer Controls a Phage Lysis-Lysogeny Decision. *Cell* **176**, 268-280.e213 (2019).

106. Wang Q, *et al.* Structural basis of the arbitrium peptide-AimR communication system in the phage lysis-lysogeny decision. *Nat Microbiol* **3**, 1266-1273 (2018).

107. Gallego Del Sol F, Penades JR, Marina A. Deciphering the Molecular Mechanism Underpinning Phage Arbitrium Communication Systems. *Mol Cell* **74**, 59-72.e53 (2019).

108. Suh GA, *et al.* Considerations for the Use of Phage Therapy in Clinical Practice. *Antimicrobial Agents and Chemotherapy* **66**, e02071-02021 (2022).

109. Azam AH, Tanji Y. Bacteriophage-host arm race: an update on the mechanism of phage resistance in bacteria and revenge of the phage with the

perspective for phage therapy. *Appl Microbiol Biotechnol* **103**, 2121-2131 (2019).

110. Fischer S, Kittler S, Klein G, Glünder G. Impact of a Single Phage and a Phage Cocktail Application in Broilers on Reduction of Campylobacter jejuni and Development of Resistance. *PLOS ONE* **8**, e78543 (2013).

111. Yang Y, *et al.* Development of a Bacteriophage Cocktail to Constrain the Emergence of Phage-Resistant Pseudomonas aeruginosa. *Frontiers in Microbiology* **11**, (2020).

112. Bernheim A, Sorek R. The pan-immune system of bacteria: antiviral defence as a community resource. *Nat Rev Microbiol* **18**, 113-119 (2020).

113. Rostøl JT, Marraffini L. (Ph)ighting Phages: How Bacteria Resist Their Parasites. *Cell Host & Microbe* **25**, 184-194 (2019).

114. Brockhurst MA, Koskella B, Zhang Q-G. Bacteria-phage antagonistic coevolution and the implications for phage therapy. *Bacteriophages: biology, technology, therapy*, 231-251 (2021).

115. Samson JE, Magadán AH, Sabri M, Moineau S. Revenge of the phages: defeating bacterial defences. *Nat Rev Microbiol* **11**, 675-687 (2013).

116. Bruynoghe R, Maisin J. Essais de thérapeutique au moyen du bacteriophage. *CR Soc Biol* **85**, 1120-1121 (1921).

117. Lavigne R, Robben J. Professor Dr. Richard Bruynoghe: A 1951 overview of his bacteriophage research spanning three decades. *Bacteriophage* **2**, 1-4 (2012).

118. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. *Bacteriophage* **1**, 66-85 (2011).

119. Marongiu L, Burkard M, Lauer UM, Hoelzle LE, Venturelli S. Reassessment of Historical Clinical Trials Supports the Effectiveness of Phage Therapy. *Clin Microbiol Rev* **35**, e00062-00022 (2022).

120. Melo LDR, Oliveira H, Pires DP, Dabrowska K, Azeredo J. Phage therapy efficacy: a review of the last 10 years of preclinical studies. *Critical Reviews in Microbiology* **46**, 78-99 (2020).

121. Gómez-Ochoa SA, *et al.* Efficacy of phage therapy in preclinical models of bacterial infection: a systematic review and meta-analysis. *The Lancet Microbe* **3**, e956-e968 (2022).

122. Rhoads DD, Wolcott RD, Kuskowski MA, Wolcott BM, Ward LS, Sulakvelidze A. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. *Journal of Wound Care* **18**, 237-243 (2009).

123. Wright A, Hawkins CH, Anggard EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. *Clin Otolaryngol* **34**, 349-357 (2009).

124. Sarker SA, *et al.* Oral Phage Therapy of Acute Bacterial Diarrhea With Two Coliphage Preparations: A Randomized Trial in Children From Bangladesh. *eBioMedicine* **4**, 124-137 (2016).

125. Jault P, *et al.* Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by Pseudomonas aeruginosa (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *Lancet Infect Dis* **19**, 35-45 (2019).

126. Uyttebroek S, *et al.* Safety and efficacy of phage therapy in difficult-to-treat infections: a systematic review. *The Lancet Infectious Diseases* **22**, e208-e220 (2022).

127. El Haddad L, Harb CP, Gebara MA, Stibich MA, Chemaly RF. A Systematic and Critical Review of Bacteriophage Therapy Against Multidrug-resistant

ESKAPE Organisms in Humans. *Clinical Infectious Diseases* **69**, 167-178 (2018).

128. Dedrick RM, *et al.* Potent antibody-mediated neutralization limits bacteriophage treatment of a pulmonary Mycobacterium abscessus infection. *Nature Medicine* **27**, 1357-1361 (2021).

129. Blasco L, *et al.* Case report: Analysis of phage therapy failure in a patient with a Pseudomonas aeruginosa prosthetic vascular graft infection. *Front Med (Lausanne)* **10**, (2023).

130. Van Nieuwenhuyse B, *et al.* A Case of In Situ Phage Therapy against Staphylococcus aureus in a Bone Allograft Polymicrobial Biofilm Infection: Outcomes and Phage-Antibiotic Interactions. *Viruses* **13**, 1898 (2021).

131. Romero FA, Razonable RR. Infections in liver transplant recipients. *World J Hepatol* **3**, 83-92 (2011).

132. Sukhum KV, *et al.* Antibiotic-resistant organisms establish reservoirs in new hospital built environments and are related to patient blood infection isolates. *Communications Medicine* **2**, 62 (2022).

133. Qin S, *et al.* Pseudomonas aeruginosa: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. *Signal Transduction and Targeted Therapy* **7**, 199 (2022).

134. Kortright KE, Chan BK, Koff JL, Turner PE. Phage Therapy: A Renewed Approach to Combat Antibiotic-Resistant Bacteria. *Cell Host Microbe* **25**, 219-232 (2019).

135. Pires DP, Boas DV, Sillankorva S, Azeredo J. Phage Therapy: a Step Forward in the Treatment of Pseudomonas aeruginosa Infections. *Journal of Virology* **89**, 7449-7456 (2015).

136. Merabishvili M, *et al.* Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. *PLoS One* **4**, e4944 (2009).

137. Lood C, *et al.* Genomics of an endemic cystic fibrosis Burkholderia multivorans strain reveals low within-patient evolution but high between-patient diversity. *PLoS Pathog* **17**, e1009418 (2021).

138. Del Barrio-Tofiño E, López-Causapé C, Oliver A. Pseudomonas aeruginosa epidemic high-risk clones and their association with horizontally-acquired β-lactamases: 2020 update. *Int J Antimicrob Agents* **56**, 106196 (2020).

139. Craig L, Forest KT, Maier B. Type IV pili: dynamics, biophysics and functional consequences. *Nat Rev Microbiol* **17**, 429-440 (2019).

140. Ceyssens PJ, *et al.* Phenotypic and genotypic variations within a single bacteriophage species. *Virol J* **8**, 134 (2011).

141. Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. *Nat Rev Microbiol* **8**, 317-327 (2010).

142. Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE. Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. *Sci Rep* **6**, 26717-26717 (2016).

143. Oechslin F, *et al.* Synergistic Interaction Between Phage Therapy and Antibiotics Clears Pseudomonas Aeruginosa Infection in Endocarditis and Reduces Virulence. *J Infect Dis* **215**, 703-712 (2017).

144. Persat A, Inclan YF, Engel JN, Stone HA, Gitai Z. Type IV pili mechanochemically regulate virulence factors in Pseudomonas aeruginosa. *Proc Natl Acad Sci U S A* **112**, 7563-7568 (2015).

145. Stewart MK, Cookson BT. Non-genetic diversity shapes infectious capacity and host resistance. *Trends in microbiology* **20**, 461-466 (2012).

146. Pirnay JP, Kutter E. Bacteriophages: it's a medicine, Jim, but not as we know it. *Lancet Infect Dis* **21**, 309-311 (2021).

147. Chanishvili N. A literature review of the practical application of bacteriophage research. *A Literature Review of the Practical Application of Bacteriophage Research*, 1-292 (2012).

148. Łusiak-Szelachowska M, *et al.* Antiphage activity of sera during phage therapy in relation to its outcome. *Future Microbiol* **12**, 109-117 (2017).

149. USSR MoHot. Ministry of Health of the USSR, Instructions of use : liquid staphylococcal phage preparation for injections (in Russian).) (1986).

150. Krut O, Bekeredjian-Ding I. Contribution of the Immune Response to Phage Therapy. *J Immunol* **200**, 3037-3044 (2018).

151. Żaczek M, *et al.* Antibody Production in Response to Staphylococcal MS-1 Phage Cocktail in Patients Undergoing Phage Therapy. *Front Microbiol* **7**, 1681 (2016).

152. Borysowski J, Gorski A. Is phage therapy acceptable in the immunocompromised host? *Int J Infect Dis* **12**, 466-471 (2008).

153. Gervassi AL, Horton H. Is Infant Immunity Actively Suppressed or Immature? *Virology (Auckl)* **2014**, 1-9 (2014).

154. Cao C, Yu M, Chai Y. Pathological alteration and therapeutic implications of sepsis-induced immune cell apoptosis. *Cell Death Dis* **10**, 782 (2019).

155. Van Belleghem JD, Clement F, Merabishvili M, Lavigne R, Vaneechoutte M. Pro- and anti-inflammatory responses of peripheral blood mononuclear cells induced by Staphylococcus aureus and Pseudomonas aeruginosa phages. *Sci Rep* **7**, 8004-8004 (2017).

156. Majewska J, *et al.* Oral Application of T4 Phage Induces Weak Antibody Production in the Gut and in the Blood. *Viruses* **7**, 4783-4799 (2015).

157. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114-2120 (2014).

158. De Coster W, D'Hert S, Schultz DT, Cruts M, Van Broeckhoven C. NanoPack: visualizing and processing long-read sequencing data. *Bioinformatics* **34**, 2666-2669 (2018).

159. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLOS Computational Biology* **13**, e1005595 (2017).

160. Tatusova T, *et al.* NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Research* **44**, 6614-6624 (2016).

161. Sedlazeck FJ, *et al.* Accurate detection of complex structural variations using single-molecule sequencing. *Nat Methods* **15**, 461-468 (2018).

162. Wick RR, Schultz MB, Zobel J, Holt KE. Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics* **31**, 3350-3352 (2015).

163. Thrane SW, Taylor VL, Lund O, Lam JS, Jelsbak L. Application of Whole-Genome Sequencing Data for O-Specific Antigen Analysis and In Silico Serotyping of Pseudomonas aeruginosa Isolates. *J Clin Microbiol* **54**, 1782-1788 (2016).

164. Huerta-Cepas J, *et al.* Fast Genome-Wide Functional Annotation through Orthology Assignment by eggNOG-Mapper. *Mol Biol Evol* **34**, 2115-2122 (2017).

165. Arndt D, *et al.* PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res* **44**, W16-21 (2016).

166. Bertelli C, *et al.* IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Res* **45**, W30-w35 (2017).

167. Hernández-Padilla L, *et al.* The Antiproliferative Effect of Cyclodipeptides from Pseudomonas aeruginosa PAO1 on HeLa Cells Involves Inhibition of Phosphorylation of Akt and S6k Kinases. *Molecules* **22**, (2017).

168. Adams MH. *Bacteriophages*. Interscience Publishers (1959).

169. Costa S, Reagan MR. Therapeutic Irradiation: Consequences for Bone and Bone Marrow Adipose Tissue. *Frontiers in Endocrinology* **10**, (2019).

170. Borrelli MR, Shen AH, Lee GK, Momeni A, Longaker MT, Wan DC. Radiation-Induced Skin Fibrosis: Pathogenesis, Current Treatment Options, and Emerging Therapeutics. *Ann Plast Surg* **83**, S59-S64 (2019).

171. Saderi H, Emadi B, Owlia P. Phenotypic and genotypic study of macrolide, lincosamide and streptogramin B (MLSB) resistance in clinical isolates of Staphylococcus aureus in Tehran, Iran. *Med Sci Monit* **17**, Br48-53 (2011).

172. Ferry T, *et al.* Phage Therapy as Adjuvant to Conservative Surgery and Antibiotics to Salvage Patients With Relapsing S. aureus Prosthetic Knee Infection. *Front Med (Lausanne)* **7**, 570572-570572 (2020).

173. Ferry T, *et al.* Case Report: Arthroscopic "Debridement Antibiotics and Implant Retention" With Local Injection of Personalized Phage Therapy to Salvage a Relapsing Pseudomonas Aeruginosa Prosthetic Knee Infection. *Front Med (Lausanne)* **8**, 569159 (2021).

174. Tkhilaishvili T, Winkler T, Müller M, Perka C, Trampuz A. Bacteriophages as Adjuvant to Antibiotics for the Treatment of Periprosthetic Joint Infection Caused by Multidrug-Resistant Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy* **64**, e00924-00919 (2019).

175. Tkhilaishvili T, Wang L, Perka C, Trampuz A, González Moreno M. Using Bacteriophages as a Trojan Horse to the Killing of Dual-Species Biofilm

Formed by Pseudomonas aeruginosa and Methicillin Resistant Staphylococcus aureus. *Frontiers in Microbiology* **11**, (2020).

176. Arenz S, *et al.* Molecular basis for erythromycin-dependent ribosome stalling during translation of the ErmBL leader peptide. *Nature Communications* **5**, 3501 (2014).

177. Syrogiannopoulos GA, *et al.* Antimicrobial susceptibility and macrolide resistance inducibility of Streptococcus pneumoniae carrying erm(A), erm(B), or mef(A). *Antimicrobial agents and chemotherapy* **47**, 2699-2702 (2003).

178. Dzyubak E, Yap MN. The Expression of Antibiotic Resistance Methyltransferase Correlates with mRNA Stability Independently of Ribosome Stalling. *Antimicrobial agents and chemotherapy* **60**, 7178-7188 (2016).

179. Walsh D, Mohr I. Viral subversion of the host protein synthesis machinery. *Nat Rev Microbiol* **9**, 860-875 (2011).

180. Rahmsdorf HJ, Herrlich P, Pai SH, Schweiger M, Wittmann HG. Ribosomes after infection with bacteriophage T4 and T7. *Molecular and General Genetics MGG* **127**, 259-271 (1973).

181. Campbell EA, *et al.* Structural mechanism for rifampicin inhibition of bacterial rna polymerase. *Cell* **104**, 901-912 (2001).

182. Vandersteegen K, *et al.* Microbiological and Molecular Assessment of Bacteriophage ISP for the Control of Staphylococcus aureus. *PloS one* **6**, e24418 (2011).

183. Becker Y. *Replication of Viral and Cellular Genomes : Molecular events at the origins of replication and biosynthesis of viral and cellular genomes*. Springer US (1983).

184. Abedon ST. Phage-Antibiotic Combination Treatments: Antagonistic Impacts of Antibiotics on the Pharmacodynamics of Phage Therapy? *Antibiotics (Basel)* **8**, 182 (2019).

185. Wang L, Tkhilaishvili T, Trampuz A, González Moreno M. Evaluation of Staphylococcal Bacteriophage Sb-1 as an Adjunctive Agent to Antibiotics Against Rifampin-Resistant Staphylococcus aureus Biofilms. *Frontiers in Microbiology* **11**, (2020).

186. Kvachadze L, *et al.* Evaluation of lytic activity of staphylococcal bacteriophage Sb-1 against freshly isolated clinical pathogens. *Microb Biotechnol* **4**, 643-650 (2011).

187. Kumaran D, *et al.* Does Treatment Order Matter? Investigating the Ability of Bacteriophage to Augment Antibiotic Activity against Staphylococcus aureus Biofilms. *Front Microbiol* **9**, 127 (2018).

188. Holland TL, Bayer AS, Fowler VG. Persistent Methicilin-Resistant Staphylococcus aureus Bacteremia: Resetting the Clock for Optimal Management. *Clin Infect Dis* **75**, 1668-1674 (2022).

189. Stacey HJ, De Soir S, Jones JD. The Safety and Efficacy of Phage Therapy: A Systematic Review of Clinical and Safety Trials. *Antibiotics* **11**, 1340 (2022).

190. Mercado-Pagán Á E, Stahl AM, Shanjani Y, Yang Y. Vascularization in bone tissue engineering constructs. *Ann Biomed Eng* **43**, 718-729 (2015).

191. Uruén C, Chopo-Escuin G, Tommassen J, Mainar-Jaime RC, Arenas J. Biofilms as Promoters of Bacterial Antibiotic Resistance and Tolerance. *Antibiotics* **10**, 3 (2021).

192. Urbina T, *et al.* Antibiotics in Necrotizing Soft Tissue Infections. *Antibiotics* **10**, 1104 (2021).

193. Köhler T, *et al.* Personalized aerosolised bacteriophage treatment of a chronic lung infection due to multidrug-resistant Pseudomonas aeruginosa. *Nature Communications* **14**, 3629 (2023).

194. Abedon ST. Lysis from without. *Bacteriophage* **1**, 46-49 (2011).

195. Dutkowski P, Linecker M, DeOliveira ML, Müllhaupt B, Clavien P-A. Challenges to Liver Transplantation and Strategies to Improve Outcomes. *Gastroenterology* **148**, 307-323 (2015).

196. Neuberger J. An update on liver transplantation: A critical review. *Journal of Autoimmunity* **66**, 51-59 (2016).

197. Yang LS, Shan LL, Saxena A, Morris DL. Liver transplantation: a systematic review of long-term quality of life. *Liver International* **34**, 1298-1313 (2014).

198. Moreno R, Berenguer M. Post-liver transplantation medical complications. *Annals of hepatology* **5**, 77-85 (2006).

199. Craig EV, Heller MT. Complications of liver transplant. *Abdominal Radiology* **46**, 43-67 (2021).

200. Choudhary NS, Saigal S, Bansal RK, Saraf N, Gautam D, Soin AS. Acute and Chronic Rejection After Liver Transplantation: What A Clinician Needs to Know. *Journal of Clinical and Experimental Hepatology* **7**, 358-366 (2017).

201. Tsinari KK, *et al.* Factors affecting metabolic and electrolyte changes after reperfusion in liver transplantation. *Transplantation Proceedings* **36**, 3051-3056 (2004).

202. Dashti SH, *et al.* Hemodynamic Changes and Early Recovery of Liver Graft Function after Liver Transplantation. *Int J Organ Transplant Med* **11**, 1-7 (2020).

203. Åberg F, Isoniemi H, Höckerstedt K. Long-Term Results of Liver Transplantation. *Scandinavian Journal of Surgery* **100**, 14-21 (2011).

204. Lerut J, Iesari S. Immunosuppression and Liver Transplantation. *Engineering* **21**, 175-187 (2023).

205. Hernandez Mdel P, Martin P, Simkins J. Infectious Complications After Liver Transplantation. *Gastroenterol Hepatol (N Y)* **11**, 741-753 (2015).

206. Kim JE, *et al*. Infections after living donor liver transplantation in children. *J Korean Med Sci* **25**, 527-531 (2010).

207. Kim SI. Bacterial infection after liver transplantation. *World J Gastroenterol* **20**, 6211-6220 (2014).

208. Shbaklo N, Tandoi F, Lupia T, Corcione S, Romagnoli R, De Rosa FG. Bacterial and Viral Infections in Liver Transplantation: New Insights from Clinical and Surgical Perspectives. *Biomedicines* **10**, (2022).

209. van Hoek B, de Rooij B-J, Verspaget HW. Risk factors for infection after liver transplantation. *Best Practice & Research Clinical Gastroenterology* **26**, 61-72 (2012).

210. Åberg F, Mäkisalo H, Höckerstedt K, Isoniemi H. Infectious Complications More Than 1 Year after Liver Transplantation: A 3-Decade Nationwide Experience. *American Journal of Transplantation* **11**, 287-295 (2011).

211. Pandanaboyana S, Bell R, Bartlett AJ, McCall J, Hidalgo E. Meta-analysis of Duct-to-duct versus Roux-en-Y biliary reconstruction following liver transplantation for primary sclerosing cholangitis. *Transpl Int* **28**, 485-491 (2015).

212. van Saene HKF, Zandstra DF. Selective decontamination of the digestive tract: Rationale behind evidence-based use in liver transplantation. *Liver Transplantation* **10**, 828-833 (2004).

213. Wiesner RH, *et al.* SELECTIVE BOWEL DECONTAMINATION TO DECREASE GRAM-NEGATIVE AEROBIC BACTERIAL AND CANDIDA COLONIZATION AND PREVENT INFECTION AFTER ORTHOTOPIC LIVER TRANSPLANTATION. *Transplantation* **45**, 570-574 (1988).

214. Steffen R, *et al.* Bacterial and fungal colonization and infections using oral selective bowel decontamination in orthotopic liver transplantations. *Transplant international* **7**, 101-108 (1994).

215. Bert F, *et al.* Pretransplant fecal carriage of extended-spectrum beta-lactamase- producing Enterobacteriaceae and infection after liver transplant, France. *Emerg Infect Dis* **18**, 908-916 (2012).

216. Hand J, Patel G. Multidrug-resistant organisms in liver transplant: Mitigating risk and managing infections. *Liver Transplantation* **22**, 1143-1153 (2016).

217. Pereira MR, *et al.* Risk factors and outcomes of carbapenem-resistant Klebsiella pneumoniae infections in liver transplant recipients. *Liver Transplantation* **21**, 1511-1519 (2015).

218. Béranger A, *et al.* Early Bacterial Infections After Pediatric Liver Transplantation in the Era of Multidrug-resistant Bacteria: Nine-year Single-center Retrospective Experience. *The Pediatric Infectious Disease Journal* **39**, 1 (2020).

219. Macesic N, *et al.* Genomic Surveillance Reveals Diversity of Multidrug-Resistant Organism Colonization and Infection: A Prospective Cohort Study in Liver Transplant Recipients. *Clinical Infectious Diseases* **67**, 905-912 (2018).

220. Bert F, *et al.* Risk factors associated with preoperative fecal carriage of extended-spectrum β-lactamase-producing E nterobacteriaceae in liver transplant recipients. *Transplant Infectious Disease* **16**, 84-89 (2014).

221. Lübbert C, *et al*. Rapid emergence of secondary resistance to gentamicin and colistin following selective digestive decontamination in patients with KPC-2-producing Klebsiella pneumoniae: a single-centre experience. *Int J Antimicrob Agents* **42**, 565-570 (2013).

222. Catho G, Huttner BD. Strategies for the eradication of extended-spectrum beta-lactamase or carbapenemase-producing Enterobacteriaceae intestinal carriage. *Expert Rev Anti Infect Ther* **17**, 557-569 (2019).

223. Mascolo A, *et al*. Decolonization of drug-resistant Enterobacteriaceae carriers: A scoping review of the literature. *Journal of Infection and Public Health* **16**, 376-383 (2023).

224. Karbalaei M, Keikha M. Probiotics and intestinal decolonization of antibiotic-resistant microorganisms; A reality or fantasy? *Ann Med Surg (Lond)* **80**, 104269 (2022).

225. Bilsen MP, Lambregts MMC, van Prehn J, Kuijper EJ. Faecal microbiota replacement to eradicate antimicrobial resistant bacteria in the intestinal tract - a systematic review. *Curr Opin Gastroenterol* **38**, 15-25 (2022).

226. Michailidis L, Currier AC, Le M, Flomenhoft DR. Adverse events of fecal microbiota transplantation: a meta-analysis of high-quality studies. *Ann Gastroenterol* **34**, 802-814 (2021).

227. Otte J-B. Pediatric liver transplantation: Personal perspectives on historical achievements and future challenges. *Liver Transplantation* **22**, 1284-1294 (2016).

228. Dalex M. Infections bactériennes survenant durant les 90 premiers jours post-greffe hépatique chez 100 enfants aux Cliniques Universitaires Saint-Luc (2012-2016). *Faculté de Médecine - Université Catholique de Louvain* **1693**, 253-268 (2018).

229. Beamud B, García-González N, Gómez-Ortega M, González-Candelas F, Domingo-Calap P, Sanjuan R. Genetic determinants of host tropism in Klebsiella phages. *Cell Rep* **42**, 112048 (2023).

230. Lourenço M, *et al.* Phages against Noncapsulated Klebsiella pneumoniae: Broader Host range, Slower Resistance. *Microbiology Spectrum* **11**, e04812-04822 (2023).

231. Rendueles O. Deciphering the role of the capsule of Klebsiella pneumoniae during pathogenesis: A cautionary tale. *Mol Microbiol* **113**, 883-888 (2020).

232. Wyres KL, Lam MMC, Holt KE. Population genomics of Klebsiella pneumoniae. *Nat Rev Microbiol* **18**, 344-359 (2020).

233. Temkin E, Margalit I, Nutman A, Carmeli Y. Surgical antibiotic prophylaxis in patients colonized with multidrug-resistant Gram-negative bacteria: practical and conceptual aspects. *J Antimicrob Chemother* **76**, i40-i46 (2021).

234. Dubinsky-Pertzov B, *et al.* Carriage of Extended-spectrum Beta-lactamase-producing Enterobacteriaceae and the Risk of Surgical Site Infection After Colorectal Surgery: A Prospective Cohort Study. *Clin Infect Dis* **68**, 1699-1704 (2019).

235. Bassis CM, *et al.* Comparison of stool versus rectal swab samples and storage conditions on bacterial community profiles. *BMC Microbiology* **17**, 78 (2017).

236. Lerner A, Romano J, Chmelnitsky I, Navon-Venezia S, Edgar R, Carmeli Y. Rectal swabs are suitable for quantifying the carriage load of KPC-producing carbapenem-resistant Enterobacteriaceae. *Antimicrob Agents Chemother* **57**, 1474-1479 (2013).

237. Siegel JD, Rhinehart E, Jackson M, Chiarello L. Management of multidrug-resistant organisms in health care settings, 2006. *American Journal of Infection Control* **35**, S165-S193 (2007).

238. Septimus EJ, Schweizer ML. Decolonization in Prevention of Health Care-Associated Infections. *Clin Microbiol Rev* **29**, 201-222 (2016).

239. Marcella C, Cui B, Kelly CR, Ianiro G, Cammarota G, Zhang F. Systematic review: the global incidence of faecal microbiota transplantation-related adverse events from 2000 to 2020. *Aliment Pharmacol Ther* **53**, 33-42 (2021).

240. Doub JB, Wilson E. Observed transaminitis with a unique bacteriophage therapy protocol to treat recalcitrant Staphylococcal biofilm infections. *Infection* **50**, 281-283 (2022).

241. Green SI, *et al.* Targeting of Mammalian Glycans Enhances Phage Predation in the Gastrointestinal Tract. *mBio* **12**, 10.1128/mbio.03474-03420 (2021).

242. Shkoporov AN, Hill C. Bacteriophages of the Human Gut: The “Known Unknown” of the Microbiome. *Cell Host & Microbe* **25**, 195-209 (2019).

243. Zuppi M, Hendrickson HL, O'Sullivan JM, Vatanen T. Phages in the Gut Ecosystem. *Front Cell Infect Microbiol* **11**, (2022).

244. Zhang Y, Wang R. The human gut phageome: composition, development, and alterations in disease. *Front Microbiol* **14**, 1213625 (2023).

245. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE. Reconstituting Organ-Level Lung Functions on a Chip. *Science* **328**, 1662-1668 (2010).

246. Huh D, *et al.* Microfabrication of human organs-on-chips. *Nature Protocols* **8**, 2135-2157 (2013).

247. Jalili-Firoozinezhad S, *et al.* A complex human gut microbiome cultured in an anaerobic intestine-on-a- chip. *Nat Biomed Eng* **3**, 520-531 (2019).

248. Chin WH, Barr J. Phage research in 'organ-on-chip. *Microbiology Australia* **40**, (2019).

249. Chin WH, *et al.* Bacteriophages evolve enhanced persistence to a mucosal surface. *Proceedings of the National Academy of Sciences* **119**, e2116197119 (2022).

250. Duyvejonck H, *et al.* Development of a qPCR platform for quantification of the five bacteriophages within bacteriophage cocktail 2 (BFC2). *Sci Rep* **9**, 13893 (2019).

251. Green SI, Kaelber JT, Ma L, Trautner BW, Ramig RF, Maresso AW. Bacteriophages from ExPEC Reservoirs Kill Pandemic Multidrug-Resistant Strains of Clonal Group ST131 in Animal Models of Bacteremia. *Sci Rep* **7**, 46151 (2017).

252. Gibson SB, *et al.* Constructing and Characterizing Bacteriophage Libraries for Phage Therapy of Human Infections. *Front Microbiol* **10**, 2537 (2019).

253. Andrews S. "FASTQC. A quality control tool for high throughput sequence data".) (2010).

254. Wick RR, Judd LM, Holt KE. Assembling the perfect bacterial genome using Oxford Nanopore and Illumina sequencing. *PLOS Computational Biology* **19**, e1010905 (2023).

255. Kolmogorov M, Yuan J, Lin Y, Pevzner PA. Assembly of long, error-prone reads using repeat graphs. *Nat Biotechnol* **37**, 540-546 (2019).

256. Wick RR, Holt KE. Polypolish: Short-read polishing of long-read bacterial genome assemblies. *PLOS Computational Biology* **18**, e1009802 (2022).

257. Zimin AV, Salzberg SL. The genome polishing tool POLCA makes fast and accurate corrections in genome assemblies. *PLoS Comput Biol* **16**, e1007981 (2020).

258. Li H, *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078-2079 (2009).

259. Schwengers O, Jelonek L, Dieckmann MA, Beyvers S, Blom J, Goesmann A. Bakta: rapid and standardized annotation of bacterial genomes via alignment-free sequence identification. *Microb Genom* **7**, (2021).

260. Robinson JT, *et al.* Integrative genomics viewer. *Nat Biotechnol* **29**, 24-26 (2011).

261. Delcher AL, Salzberg SL, Phillippy AM. Using MUMmer to Identify Similar Regions in Large Sequence Sets. *Current Protocols in Bioinformatics* **00**, 10.13.11-10.13.18 (2003).

262. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068-2069 (2014).

263. Page AJ, *et al.* Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* **31**, 3691-3693 (2015).

264. Wickham H, *et al.* Welcome to the Tidyverse. *Journal of Open Source Software* **4**, 1686 (2019).

265. Gu Z, Gu L, Eils R, Schlesner M, Brors B. circlize Implements and enhances circular visualization in R. *Bioinformatics* **30**, 2811-2812 (2014).

266. Van Nieuwenhuyse B, *et al.* Bacteriophage-antibiotic combination therapy against extensively drug-resistant Pseudomonas aeruginosa infection to allow liver transplantation in a toddler. *Nature Communications* **13**, 5725 (2022).

267. Community TG. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update. *Nucleic Acids Res* **50**, W345-w351 (2022).

268. Okonechnikov K, Golosova O, Fursov M. Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics* **28**, 1166-1167 (2012).

269. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9**, 357-359 (2012).

270. Barr JJ, *et al.* Bacteriophage adhering to mucus provide a non-host-derived immunity. *Proc Natl Acad Sci U S A* **110**, 10771-10776 (2013).

271. Barr JJ, *et al.* Subdiffusive motion of bacteriophage in mucosal surfaces increases the frequency of bacterial encounters. *Proc Natl Acad Sci U S A* **112**, 13675-13680 (2015).

272. Almeida GMF, Laanto E, Ashrafi R, Sundberg LR. Bacteriophage Adherence to Mucus Mediates Preventive Protection against Pathogenic Bacteria. *mBio* **10**, (2019).

273. Fraser JS, Yu Z, Maxwell KL, Davidson AR. Ig-like domains on bacteriophages: a tale of promiscuity and deceit. *J Mol Biol* **359**, 496-507 (2006).

274. Fraser JS, Maxwell KL, Davidson AR. Immunoglobulin-like domains on bacteriophage: weapons of modest damage? *Curr Opin Microbiol* **10**, 382-387 (2007).

275. Bu XD, Li N, Tian XQ, Huang PL. Caco-2 and LS174T cell lines provide different models for studying mucin expression in colon cancer. *Tissue Cell* **43**, 201-206 (2011).

276. Jalili-Firoozinezhad S, *et al.* Modeling radiation injury-induced cell death and countermeasure drug responses in a human Gut-on-a-Chip. *Cell Death Dis* **9**, 223 (2018).

277. Johansson ME, Larsson JM, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. *Proc Natl Acad Sci U S A* **108 Suppl 1**, 4659-4665 (2011).

278. Le S, *et al.* Chromosomal DNA deletion confers phage resistance to Pseudomonas aeruginosa. *Sci Rep* **4**, 4738 (2014).

279. Chen C, Yang X, Shen X. Confirmed and Potential Roles of Bacterial T6SSs in the Intestinal Ecosystem. *Frontiers in Microbiology* **10**, (2019).

280. Liu Y. A code within the genetic code: codon usage regulates co-translational protein folding. *Cell Communication and Signaling* **18**, 145 (2020).

281. Grocock RJ, Sharp PM. Synonymous codon usage in Pseudomonas aeruginosa PA01. *Gene* **289**, 131-139 (2002).

282. Menon ND, *et al.* Increased Innate Immune Susceptibility in Hyperpigmented Bacteriophage-Resistant Mutants of Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **66**, e0023922 (2022).

283. Chibani-Chennoufi S, Sidoti J, Bruttin A, Kutter E, Sarker S, Brüssow H. In Vitro and In Vivo Bacteriolytic Activities of Escherichia coli Phages: Implications for Phage Therapy. *Antimicrobial Agents and Chemotherapy* **48**, 2558-2569 (2004).

284. Lourenço M, *et al.* The gut environment regulates bacterial gene expression which modulates susceptibility to bacteriophage infection. *Cell Host & Microbe* **30**, 556-569.e555 (2022).

285. Lourenço M, *et al.* The Spatial Heterogeneity of the Gut Limits Predation and Fosters Coexistence of Bacteria and Bacteriophages. *Cell Host Microbe* **28**, 390-401.e395 (2020).

286. Brown M, Hall A, Zahn H, Eisenberg M, Erickson S. Bacteriophage-Based Detection of Staphylococcus aureus in Human Serum. *Viruses* **14**, 1748 (2022).

287. Takeuchi I, Osada K, Azam AH, Asakawa H, Miyanaga K, Tanji Y. The Presence of Two Receptor-Binding Proteins Contributes to the Wide Host

Range of Staphylococcal Twort-Like Phages. *Applied and Environmental Microbiology* **82**, 5763-5774 (2016).

288. Bull JJ, Vimr ER, Molineux IJ. A tale of tails: Sialidase is key to success in a model of phage therapy against K1-capsulated Escherichia coli. *Virology* **398**, 79-86 (2010).

289. Knezevic P, *et al.* Phages of Pseudomonas aeruginosa: response to environmental factors and in vitro ability to inhibit bacterial growth and biofilm formation. *Journal of Applied Microbiology* **111**, 245-254 (2011).

290. Hodyra-Stefaniak K, *et al.* Mammalian Host-Versus-Phage immune response determines phage fate in vivo. *Sci Rep* **5**, 14802 (2015).

291. Schumann AR, Sue AD, Roach DR. Hypoxia Increases the Tempo of Phage Resistance and Mutational Bottlenecking of Pseudomonas aeruginosa. *Front Microbiol* **13**, 905343 (2022).

292. Bichet MC, *et al.* Bacteriophage uptake by mammalian cell layers represents a potential sink that may impact phage therapy. *iScience* **24**, 102287 (2021).

293. Gogokhia L, Round JL. Immune-bacteriophage interactions in inflammatory bowel diseases. *Curr Opin Virol* **49**, 30-35 (2021).

294. Sausset R, Petit MA, Gaboriau-Routhiau V, De Paepe M. New insights into intestinal phages. *Mucosal Immunology* **13**, 205-215 (2020).

295. Roach DR, *et al.* Synergy between the Host Immune System and Bacteriophage Is Essential for Successful Phage Therapy against an Acute Respiratory Pathogen. *Cell Host Microbe* **22**, 38-47.e34 (2017).

296. Forsyth JH, *et al.* Decolonizing drug-resistant E. coli with phage and probiotics: breaking the frequency-dependent dominance of residents. *Microbiology (Reading)* **169**, (2023).

297. Vashisth M, Yashveer S, Anand T, Virmani N, Bera BC, Vaid RK. Antibiotics targeting bacterial protein synthesis reduce the lytic activity of bacteriophages. *Virus Res* **321**, 198909 (2022).

298. Zuo P, Yu P, Alvarez PJJ. Aminoglycosides Antagonize Bacteriophage Proliferation, Attenuating Phage Suppression of Bacterial Growth, Biofilm Formation, and Antibiotic Resistance. *Applied and Environmental Microbiology* **87**, e00468-00421 (2021).

299. Pirnay J-P, *et al.* Retrospective, observational analysis of the first one hundred consecutive cases of personalized bacteriophage therapy of difficult-to-treat infections facilitated by a Belgian consortium. *medRxiv*, 2023.2008.2028.23294728 (2023).

300. Federici S, *et al.* Targeted suppression of human IBD-associated gut microbiota commensals by phage consortia for treatment of intestinal inflammation. *Cell* **185**, 2879-2898.e2824 (2022).

301. Fujiki J, Schnabl B. Phage therapy: Targeting intestinal bacterial microbiota for the treatment of liver diseases. *JHEP Rep* **5**, 100909 (2023).