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**“Establishing the Parameters of Host-
Pathogen Interactions in Pyogenic
Liver Abscess caused by Hypervirulent
Klebsiella pneumoniae.”**

Martin François Robert Vandeputte



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Supervisors: Thamarai Schneiders, John Plevris, David Dockrell.

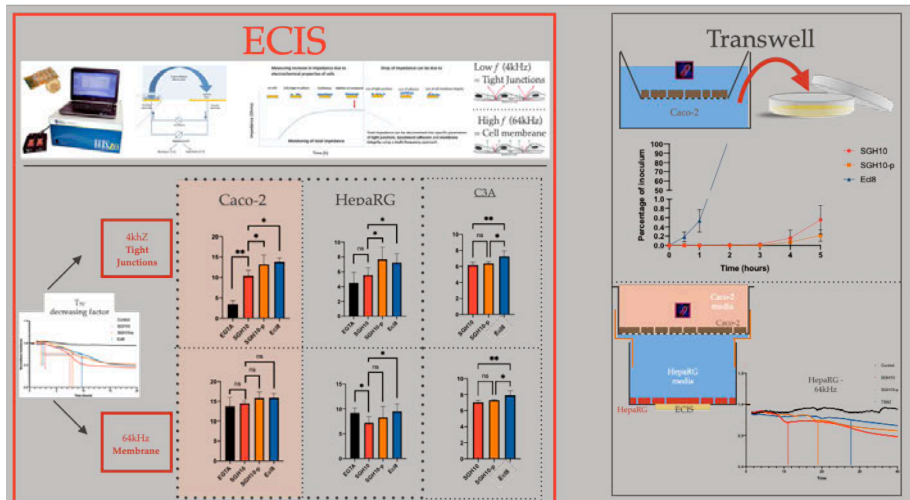
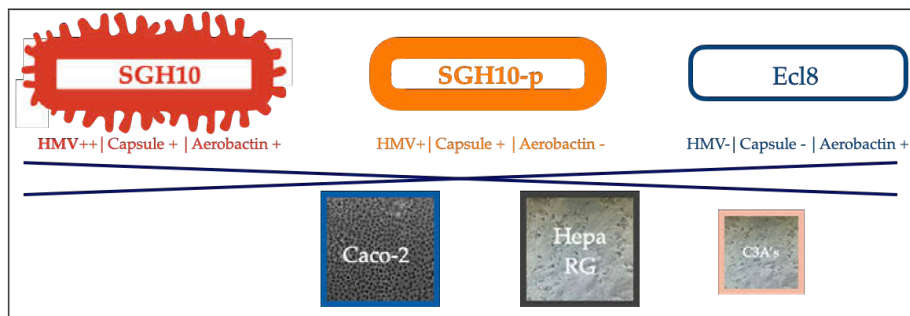
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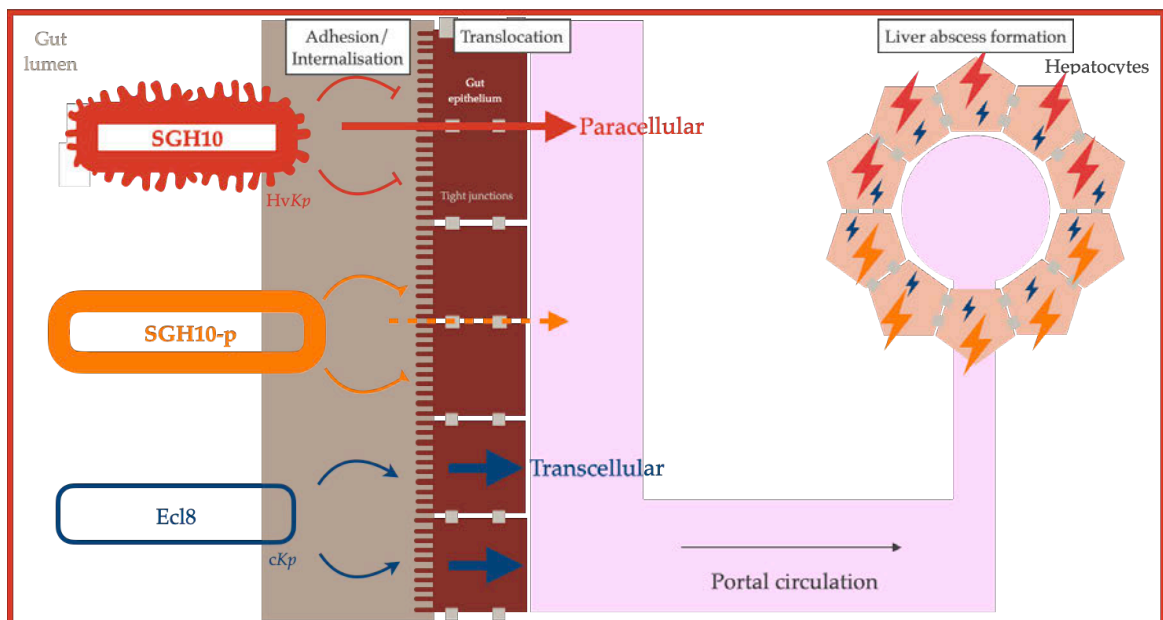
Martin François Robert Vandeputte

University of Edinburgh

Graphical abstract



General model



Abstract

Klebsiella pneumoniae (*Kp*) is a Gram-negative bacterium, classically known to cause opportunistic infections in patients with comorbidities. Since the 1980s, strains of *Kp* causing pyogenic liver abscess, with dissemination to other tissues (brain, lungs, kidneys, etc.) in young and immunocompetent people have been described as "hypervirulent" (Hv*Kp*).

Beyond its clinical definition, the main microbiological characteristics that give Hv*Kp* its hypervirulent nature are a thick capsule, hypermucoviscosity (HMV) and the production of siderophores, molecules that help capturing iron necessary for infection. Encoded by various genes (subsequently associated with hypervirulence), these phenotypes have mainly been shown to enable Hv*Kp* to survive in adverse environments (such as the human body) and to avoid the immune system.

However, their precise role in Hv*Kp*'s infection process is currently unclear.

It has been shown that Hv*Kp* first colonises the intestine, before crossing the epithelial barrier to reach the portal system and form an abscess in the liver, but the mechanism of this switch from "commensal in the intestine" to "pathogenic in the liver" is unknown. The hypothesis of this PhD project was therefore that Hv*Kp* presented specific interactions along the gut-liver axis, both to cross the gut barrier and to damage liver cells.

The aim of this work was to verify this hypothesis by developing and validating an *in vitro* infection model to (1) explore the nature, interconnections and mechanisms of the interactions between gut/liver cells and Hv*Kp* and (2) establish the role of key microbe-associated virulence factors in the pathophysiology of these liver abscesses.

This model was based on ECIS (Electrical Cell Impedance System), a platform measuring the electrical resistance generated by a layer of eukaryotic cells, and its

evolution in response to external stimuli (bacteria in our case) over time. Remarkably, this resistance is recorded at different current frequencies, representing various parameters: low frequencies (4kHz) mainly reflect tight junction (TJ) integrity and higher frequencies (64kHz) cell membrane integrity.

Cell lines representative of tissues of the gut-liver axis involved in the pathophysiological process of *HvKp* (Caco-2 for intestinal cells, HepaRG for liver cells and HUVECS for endothelial cells) were infected with different strains of *HvKp*, varying in their hypervirulent characteristics. These strains all to were SGH10, a classical *HvKp* strain belonging to the clonal group 23, SGH10 without its virulence plasmid (SGH10-p), and Ecl8, a strain belonging to a clonal group associated with hypervirulence but with attenuated virulence.

The first stage of our work involved characterising these strains, in terms of their bacterial phenotype, their interactions with eukaryotic cells and their lethality in a zebrafish larvae infection model. We were able to confirm that SGH10 was hypercapsulated, HMV and secreted siderophores; SGH10-p had lost this HMV characteristic and the secretion of siderophores; while Ecl8 was neither HMV nor hypercapsulated, but secreted siderophores. These strains' specific characteristics were reflected in their interactions with gut and liver cells, with SGH10 adhering very poorly to epithelial cells, avoiding immune cells and significantly disseminating and killing zebrafish larvae. These results also demonstrate that HMV and hypercapsulation were key factors responsible for these interactions.

These differences between our strains allowed us to use them with confidence in our ECIS model. By first infecting intestinal Caco-2 cells, we demonstrated that SGH10 specifically disrupted the intestinal tight junctions more rapidly (up to 4 hours earlier)

relative to SGH10-p and Ecl8, indicating a major role for HMV in this process. In the liver, the capsule seemed to be the main pathophysiological factor as SGH10 and SGH10-p both had a greater detrimental effect on the cell membrane than Ecl8.

All these findings were substantiated using alternative methods: the impact of SGH10 on intestinal TJs was confirmed using a TEER (Trans-epithelial electrical resistance) system, while the viability of liver cells was verified using flow cytometry. By studying the expression of TJ's proteins (claudins, occludin, ZO-1), we were able to show that *HvKp* specifically downregulated claudin-1 and occludin expression, two proteins critical for epithelial integrity. Next, by adapting and developing our model, we were also able to show that these *HvKp* effects were specific to the gut-liver axis, that they required the presence of living bacteria, and that they were not linked to secreted factors or to the epithelial cells' immune response.

We were finally able to show that, while there was no clear communication between the intestine and the liver, the HMV and hypercapsulated nature of *HvKp* meant that it passed through the intestinal epithelium using a paracellular route, taking advantage of the disruption of the tight junctions. Crucially, this preserved its subsequent toxicity on liver cells. Conversely, the strains that were neither HMV nor hypercapsulated were internalised in the intestinal cells, thus passing by a transcellular route, which was faster but resulted in a significantly reduced toxicity in the liver.

Overall, our model enabled us to describe for the first time that *HvKp* uses both its HMV and hypercapsule phenotype to specifically avoid contact with eukaryotic cells, allowing it to disrupt specific TJ proteins by downregulating claudin-1 and occludin, to

pass through the intestinal epithelium via a paracellular route and to retain its significant toxicity when reaching the liver.

Lay abstract

Bacteria, the oldest living organisms on Earth, have close and complex relationships with humans. One of the most fascinating sites in terms of these interactions is the intestine. When bacteria reach the gut of their human hosts, they can either colonise it, i.e. settle without causing any harm (and often even benefiting the host), or lead to an infection, either locally or at a distance after migration through the intestinal wall. This outcome is determined by the fine balance between the host's local defences and the bacterial mechanisms used to counter them. The main gut defence mechanism preventing resident bacteria from evading to distant tissues is a structure called epithelium, composed of a layer of cells firmly attached to each other forming a continuous barrier. This is crucial as certain bacteria, known as "opportunists", may be initially contained in the gut by its various lines of protection, before taking advantage of a breakdown in these to cause an infection.

One such bacterium is *Klebsiella pneumoniae*, representing a classical cause of hospital-acquired infections in fragile patients. In the 1980s, a new variant of this species was observed and called "hypervirulent" ("HvKp"), precisely because it does not need this weakening of the patient's defences to cause an infection. Increasingly described, initially in Asia and now globally, the main clinical manifestation are abscesses in the liver with potential dissemination to other sites such as the lungs and the brain. Interestingly, HvKp have also been shown to colonise the host intestine years before causing a liver abscess. The reason why these bacteria succeed in generating an infection "by themselves" are linked to their specific characteristics. For example, they have a very thick, sticky outer envelope (capsule), which enables them to avoid being recognised by the human's immune cells. Yet, the precise mechanisms by which they remain in the gut for so long and then eventually cross this intestinal

barrier and reach the liver are unknown. It is also unclear how they further damage liver cells to form an abscess.

The hypothesis of our study was that these *HvKp* exhibit specific interactions with the intestinal epithelium and the liver tissue. To prove this hypothesis, we developed a laboratory-based infection model using a method called ECIS (Electrical Cell Impedance System), which measures the electrical resistance generated by a layer of epithelial cells. By applying various parameters, this method enables us to precisely study the action of an external agent (bacteria in our case) on these cell lines. We infected various relevant cell lines (intestinal, hepatic, but also blood vessels) with bacteria carefully selected for their different levels and factors of virulence.

Using this setup, we have shown that *HvKp* has a particular interaction with intestinal and hepatic cells. In the intestine, *HvKp* destroyed specific proteins that enable epithelial cells to hold together (part of what is called the “tight junctions”), while in the liver *HvKp* destroyed the membrane of hepatic cells. We were also able to observe that it was the living bacteria which exerted this effect, therefore not linked to any other external factor that they can sometimes secrete. Furthermore, these interactions were only observed with liver and intestinal cells, reinforcing their value and specificity in the *HvKp*’s infection process.

Finally, by studying the interconnections of the infection process between the intestine and the liver, we were able to establish an overall mechanism by which *HvKp* crossed the intestine and created an abscess in the liver: it remains outside the cells of the intestine, which enables it to damage the tight junctions, and finally passes between the cells (unlike certain bacteria which are engulfed and pass through the cells). This

takes longer but allows the bacteria to retain their pathogenic mechanisms intact, as illustrated by their conserved harmful effect on the liver cells.

In conclusion, we have succeeded in developing a set of experiments using various technologies to elucidate how *HvKp* creates an abscess in the liver from its reservoir in the intestine. This will hopefully enable future research to attempt to inhibit this process, and eventually find a treatment for this serious disease.

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Acronyms

ALD	Alcoholic liver disease
AUC	Area under the curve
BCA	Bicinchoninic acid assay
CAS	Chrome azurol s
CFU	Colony-forming unit
CG	Clonal group
cKp	Classical <i>Klebsiella pneumoniae</i>
Cm	Chloramphenicol
CNS	Central nervous system
Cps	Capsular polysaccharide synthesis
Ct	Cycle treshold
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECIS	Electrical cell impedance system
EGM	Endothelial cell growth medium
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
EPEC	Enteropathogenic <i>Escherichia coli</i>
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
GFP	Green-fluorescent protein
GPS	General purposed supplement
HAI	Hospital-acquired infection
HLA	Human leukocyte antigen
HMV	Hypermucoviscosity
HUVEC	Human umbilical vein endothelial cells
HvKp	Hypervirulent <i>Klebsiella pneumoniae</i>
HvKp-PLA	Hypervirulent <i>Klebsiella pneumoniae</i> pyogenic liver abscess
ICE	Integrative and conjugative element
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JAM	Junctional adhesion molecules
Km	Kanamycin
Kp	<i>Klebsiella pneumoniae</i>
LB	Luria-Bertani broth
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MDCK	Madin-Darby canine kidney
MDM	Monocytes-derived macrophages
MDR	Multidrug resistant
MEME	Modified essential medium, Eagle

MLCK	Myosin light chain kinases
MMM	Maintenance & metabolism medium
MOI	Multiplicity of infection
NAFLD	Non-alcoholic fatty liver disease
OD	Optical density
OM	Outer membrane
PBMC	Human peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PLA	Pyogenic liver abscess
PTU	Phenylthiourea
qPCR	Quantitative PCR
RFP	Red fluorescent protein
<i>rmp</i>	Regulator of mucoid phenotype
RNA	Ribonucleic acid
SO	Super Optimal broth with Catabolite repression
ST	Sequence type
T₅₀DF	T50 decreasing factor
TC	Tissue culture
TEER	Trans-epithelial electrical resistance
TJ	Tight junction
TLR	Toll-like receptor
TNF	Tumor necrosis factor
ZF	Zebrafish
ZFL	Zebrafish larvae
ZO	Zona occludens

Contents

Graphical abstract.....	I
Abstract	II
Lay abstract	VI
Acknowledgments.....	IX
Acronyms	XII
Contents	XIV
Chapter 1: Introduction	1
1.1 Bacteria and the human host, commensalism and infection	1
1.2 Interactions between bacteria and host's epithelial cells	6
1.2.1 Description and structure of epithelial cell layer	6
1.2.2 Epithelial cell layer integrity and tight junctions	8
1.2.3 Adhesion of bacteria to epithelial cells.....	11
1.2.4 Epithelial and mucosal immune systems.....	13
1.2.5 Passage of bacteria through epithelium	15
1.3 Bacteria along the gut-liver axis.....	18
1.3.1 The gut-liver axis.....	18
1.3.1.1 Anatomical description	18
Role of the gut-liver axis	20
1.3.2 Pathophysiological implications	21
1.3.2.1 Role of the gut microbiota in liver diseases.....	21
1.3.2.2 Gut commensal and opportunistic bacteria.....	22
1.3.2.3 Bacterial infections of the liver	23
1.4 Classical <i>Klebsiella pneumoniae</i> , a common opportunistic pathogen	26
1.5 Hypervirulent <i>K.pneumoniae</i>	28
1.5.1 Background.....	28
1.5.2 Delineation of HvKp and cKp	32
1.5.3 Kp-ubiquitous virulence factors.....	35
1.5.4 HvKp-specific virulence factors.....	38
1.5.4.1 Capsule – Hypermucoviscosity	40
1.5.4.2 Siderophores.....	44
1.5.4.3 Colibactin.....	44
1.5.5 HvKp and antimicrobial resistance	46
1.5.6 (Hv)Kp and the immune system.....	49
1.6 Pathophysiology of HvKp-PLA.....	52
1.6.1 Gut colonisation.....	52

1.6.2	Translocation into the portal bloodstream	53
1.6.3	Access to the liver and abscess development.....	56
1.7	<i>In vitro</i> model of HvKp to mimick infection along the gut-liver axis.....	57
1.7.1	ECIS.....	57
1.7.2	Experimental cell lines of the gut-liver axis model	61
1.7.2.1	HepaRG.....	61
1.7.2.2	Caco-2	61
1.7.2.3	C3A	62
1.7.2.4	HUVEC	62
1.8	The zebrafish model	64
1.9	Aims/hypothesis	67
1.9.1	Hypothesis	67
1.9.2	Aims	67
Chapter 2:	Material & Methods.....	69
2.1	Bacterial work	69
2.1.1	Bacterial Growth Media	69
2.1.1.1	LB agar	69
2.1.1.2	M9 minimal media.....	69
2.1.2	Storage of strains.....	69
2.1.3	Bacterial strains used	70
2.1.4	Primers & Plasmids	73
2.1.5	Antibiotics	74
2.1.6	Plasmid removal protocol.....	74
2.1.7	Growth curves of bacteria.....	74
2.1.7.1	Relation between optical density (OD ₆₀₀) and bacterial growth (cfu/ml).....	74
2.1.7.2	Growth in tissue culture media/PBS.....	75
2.1.8	Quantification of capsule production	76
2.1.8.1	Percoll stage	76
2.1.8.2	Uronic acid assay quantification	76
2.1.9	Quantification of mucoviscosity levels	77
2.1.10	Chrome Azurol S (CAS) experiments to determine siderophores production	78
2.1.11	RNA extraction and Quantitative real time PCR (qPCR)	78
2.1.12	Genetic manipulation of our strains.....	79
2.1.12.1	Q5 High Fidelity PCR	82
2.1.12.2	OneTaq colony PCR.....	83
2.1.12.3	Agarose Gel Electrophoresis.....	83
2.1.12.4	Purification of PCR/Restriction Digested DNA.....	84

2.1.12.5	Preparation of calcium competent DH10 β cells.....	84
2.1.12.6	Preparation of HvKp electrocompetent cells	84
2.1.12.7	Plasmid DNA Extraction.....	85
2.1.12.8	Plasmid transformation.....	85
a.	Heat shock for DH10 β	85
b.	Electroporation	85
2.2	Cell culture	87
2.3	Infection assays.....	89
2.3.1	Determination of the multiplicity of infection (MOI)	89
2.3.2	Adhesion assay	90
2.3.3	Invasion assay.....	90
2.3.4	Microscopy (EVOS) assay.....	91
2.3.4.1	Kp-eukaryotic cells interactions visualisation.....	91
2.3.4.2	Assessment of tight junctions.....	91
2.3.5	ECIS.....	92
2.3.5.1	Simple infection model	92
2.3.5.2	Infection with heat-inactivated or filtered bacteria	94
2.3.5.3	Interrupted infection	94
2.3.5.4	Transfer of supernatant from infected Caco-2 cells	95
2.3.5.5	Transwell model	95
2.3.6	Simple Transwell model.....	96
2.3.7	TEER (TransEpithelial Electrical Resistance).....	96
2.3.8	PrestoBlue assay	97
2.3.9	LDH secretion measurement.....	98
2.3.10	CellTox™ Green Cytotoxicity Assay	98
2.3.11	Cell viability by flow cytometry	98
2.4	Innate immune response assessment.....	100
2.4.1	Cytokine secretion	100
2.4.2	Chemotaxis assessment using ECIS.....	101
2.4.2.1	Isolation of human peripheral blood mononuclear cells (PBMC) and culturing into monocytes-derived macrophages (MDM).....	101
2.4.2.2	Chemotaxis assay using ECIS	101
2.5	Western blotting for Caco-2 cells tight junction proteins	103
2.6	Zebrafish infection.....	105
2.6.1	Collection and preparation of zebrafish embryos.....	105
2.6.2	Infection by immersion	106
2.6.2.1	Survival rate	106

2.6.2.2	Dissemination rates	106
2.6.2.3	Imaging	106
2.7	Statistical analysis.....	107
Chapter 3: Characterisation of <i>Kp</i> 's interactions with eukaryotic cells and role of the virulence plasmid.....		108
3.1	Introduction	108
3.2	Results.....	112
3.2.1	Growth curves of bacteria	112
3.2.1.1	Growth of the strains in LB broth and eukaryotic tissue culture media.....	112
3.2.1.2	Relationship between optical density (OD) and bacterial growth (cfu/ml).....	115
3.2.2	Capsule production and hypermucoviscosity	117
3.2.3	Siderophores production assessment using CAS plates	119
3.2.4	qPCR for <i>rmpA/C</i> , <i>iuc</i> and capsule genes <i>wzi</i> and <i>wcaJ</i>	122
3.2.5	Eukaryotic cells count for determination of the MOI	126
3.2.6	Adhesion and internalisation of our strains to intestinal, hepatic and endothelial cells.....	127
3.2.7	Qualitative visualisation of adhesion to eukaryotic cells using immunohistochemistry	129
3.2.7.1	Electroporation of pRSMGFP+ in SGH10:	129
3.2.7.2	Visualisation of images.....	130
3.2.8	Interactions with human Monocyte-Derived Macrophages (MDM) using the ECIS system	132
3.2.9	<i>In vivo</i> assessment of virulence using the zebrafish model.....	134
3.2.9.1	Survival after infection by immersion	134
3.2.9.2	Bacterial dissemination after infection by immersion.....	135
3.3	Discussion.....	136
Chapter 4: Implementation of an impedance assay-based model for studying host-pathogens interactions between gut/liver cells and <i>HvKp</i>		141
4.1	Introduction	141
4.2	Results.....	144
4.2.1	Monitoring of host-pathogen interactions between gut, liver and endothelial cells and <i>HvKp</i> using the impedance-based ECIS assay.....	144
4.2.1.1	Direct infection on ECIS	144
4.2.1.2	Normalisation of the data and determination of T50 decrease factor 146	
4.2.1.3	<i>HvKp</i> specifically disrupt gut cells TJ	150
4.2.1.4	The thick capsule potentiates damages to hepatocytes.....	150
4.2.1.5	<i>HvKp</i> has no specific effect on HUVECS cells or bronchial epithelial cells.	151
4.2.1.6	These results were reproducible with another <i>HvKp</i> strain	152

4.2.2	Confirmation of the gut TJ greater disruption by <i>HvKp</i>	154
4.2.2.1	Using ICC	154
4.2.2.2	Using TEER	157
4.2.3	Confirmation of liver cell toxicity.....	159
4.2.3.1	PrestoBlue, LDH secretion and CellTox cytotoxicity assays	159
4.2.3.2	Flow cytometry	160
4.3	Discussion.....	164
Chapter 5: Further characterisation of the damage and disruption of epithelial cells in colonisation and pathogenesis by <i>HvKp</i>		169
5.1	Introduction	169
5.2	Results.....	172
5.2.1	<i>HvKp</i> 's toxicity on gut and liver cells necessitates live extracellular bacteria.....	172
5.2.2	Assessment of eukaryotic cell cytokine secretion	176
5.2.3	Characteristics and communication of <i>HvKp</i> infection along the gut-liver axis.....	178
5.2.3.1	Incubation of HepaRG with supernatant of infected Caco2.....	178
5.2.3.2	Transwell system on ECIS.....	180
5.2.4	Particular effect of <i>HvKp</i> on intestinal TJ proteins	183
5.2.5	Role of <i>HvKp</i> 's specific HMV and hypercapsule genes to this pathophysiology	186
5.3	Discussion.....	187
Chapter 6: General discussion		191
Chapter 7: Limitations and future work.....		195
References.....		197
Appendix		235

Chapter 1: Introduction

1.1 Bacteria and the human host, commensalism and infection

The links between microbes and humans are numerous, complex, and fascinating. The main interaction of microorganisms with the human body is their ability to colonise it, i.e. access and establish themselves on or within many of its different systems. As described in detail in this chapter, these colonisers can develop different relationships with the human body: they can (1) merely reside there, without causing any damage or interaction, (2) develop a symbiotic relationship (known as commensalism), or (3) exercise pathogenic power (as either opportunistic or obligate pathogens).

Whilst many bacteria will settle in the human body without any effect (either harmful or beneficial), the simplest and oldest relationship is the one that allows the bacterium to benefit from its presence in its host's body, known as "commensalism". This word comes from the Latin « cum » (with) and « mensa » (table): "who eat at the same table", designating a symbiotic and synergetic relationship. These organisms are normal inhabitants of the human body, present in different systems, e.g. in the gastrointestinal or genital tracts, on the skin or in the lungs, living in communities called "microbiota", together forming what is known as the microbiome. Strikingly, they outnumber human cells; it is estimated, for example, that there are 10^{12} bacteria per gram of colon.^{1,2} The human host supports microbial survival and growth by offering a safe habitat with nutrients and growth factors.

The microbiota is also, by definition, beneficial to the human host, in many ways: it helps with the extraction of energy, nutrients and micronutrients (e.g. vitamins) from

food, enhances the differentiation of mucosal structures, has a significant immunoregulatory role and a physical barrier function, providing “colonisation resistance” against pathogenic microbes.³ *Lactobacillus* species, for instance, can attach to the gut surface and process undigested carbohydrates, producing energy-rich compounds that are further absorbed to the portal circulation.⁴ The microbiota maintains its functions and viability by means of a subtle balance between the different bacterial species that make it up.

Remarkably, when a colonising and/or a commensal bacterium from the microbiota either migrates to another location or outnumbers the other species, it can become pathogenic to their host. These microorganisms are called "opportunistic pathogens": they do not have an inherent ability to breach the host's barriers, and the disease they cause only results from an “opportunity”, i.e. a weakening of the host's defences, allowing this migration and/or proliferation.⁵ In the intestine for example, as summarised in **Figure 1.1**, damage to the epithelium, reduced peristalsis, destruction of the microbiota by antibiotics, decreased mucus secretion or increased pH can all result in the proliferation and translocation of colonising or commensal bacteria, becoming “pathogens” de facto.

This will lead to an infection, which can be defined as a medical condition following the invasion and multiplication of microorganisms within the host, either in an unusual site or in number. Both specific pathogen toxicity factors and the host's immune response further disrupt the normal functioning of the tissues and organs, causing a variety of clinical manifestations, potentially leading to death. A good example is *Escherichia coli* (*E. coli*), a common commensal bacterium residing in the human gut, playing a

beneficial role in digestion but potentially becoming an opportunistic pathogen if it translocates from the gut to other body sites. This migration involves alterations in the host's normal defences as described above but, once outside the gut, *E. coli* can produce virulence factors like adhesins and toxins, leading for instance to infections in the urinary tract or bloodstream.⁶ *Pseudomonas aeruginosa* is not designated as commensal as it typically colonises healthy individuals without harm, but without benefits either. Yet, it is also an opportunistic pathogen as it causes a wide variety of infections when an opportunity arises, e.g. in immunocompromised patients or those with cystic fibrosis.⁷

The second major pathophysiological mechanism resulting in an infection involves bacteria designated as either “facultative” or “obligate” pathogens. These are meant to cause an infection, and rarely colonise the host without causing any harm. Obligate must exploit their host to complete their life cycle and cannot survive or reproduce outside a host, while facultative pathogens can replicate in the environment reservoir, causing disease when reaching a susceptible host. Both have evolved specific strategies and possess virulence factors, which provide them with the ability to breach the host's epithelia or cause cell toxicity, leading to an infection. These virulence factors can for instance be responsible for immune evasion, toxin secretion damaging host cells, resistance to hostile environments or increased adhesion to epithelia (**Figure 1.1**).

An example of a facultative pathogen is *Vibrio cholerae*, the causative agent of cholera, a Gram-negative bacterium found in aquatic environments and infecting humans through contaminated water and food. Once inside the host's small intestine, *Vibrio*

cholerae produces cholera toxin (virulence factor), which binds to intestinal epithelial cells, causing them to release excessive fluid and electrolytes into the gut lumen, resulting in profuse diarrhoea.^{8,9}

Notably, whilst the relations between the human host and bacteria are variable, the distinction between coloniser, commensal, opportunistic, facultative or obligate pathogen is often blurred. For instance, obligate pathogens can colonise the host for a long period of time, sometimes even playing a part in a microbiota (therefore behaving as commensals) before eventually causing a disease. Typical examples are *Streptococcus pneumoniae* or *Neisseria meningitidis*, known to colonise the upper respiratory tract but potentially causing disease both in immunocompetent (behaving like obligate pathogen) and immunocompromised hosts (behaving like opportunistic pathogens).¹⁰

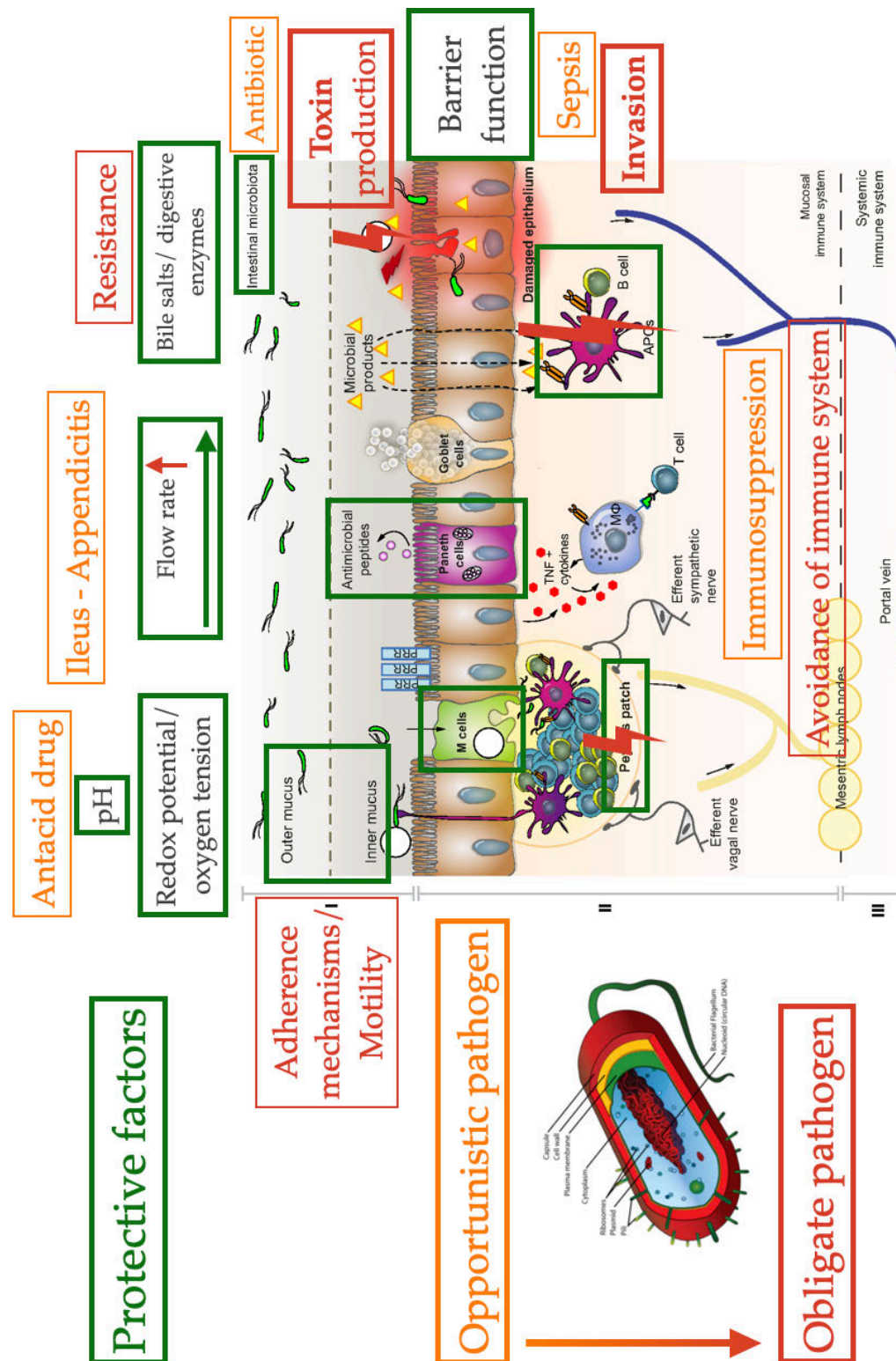


Figure 1.1 Schematic representation of the protective factors of the intestinal epithelium (framed in green), and the mechanisms leading to infection by opportunistic (orange) or obligate (red) pathogens. Figure adapted from ¹¹

1.2 Interactions between bacteria and host's epithelial cells

1.2.1 Description and structure of epithelial cell layer

The epithelium is a complex but fundamental tissue covering external and internal surfaces of organs of the human body. As illustrated in **Figure 1.2**, it is composed of tightly attached cells, forming sheets that can be simple (single layer) or stratified (multiple layers), and coming in different shapes (squamous, cuboidal or columnar). Whilst epithelia play crucial roles in absorption (as in the gut), secretion (glandular tissue) and transport (respiratory tract cilia), their most critical function in the context of the host-pathogen interactions is their barrier function. Epithelial cells surfacing organs in direct or indirect contact with the environment, such as the skin or the digestive and respiratory systems, are likely to be the first encounter between bacteria and the human body. These are therefore a major component of the body's defence mechanisms, safeguarding internal tissues from bacterial invasion, that will either be cleared or adhere to them.^{12,13}

As described in detail below, the adhesion of the microorganisms to the epithelium can be the destination of the bacterium (e.g. before colonising a microbiota), whilst some pathogenic bacteria can also seek to cross this epithelium.

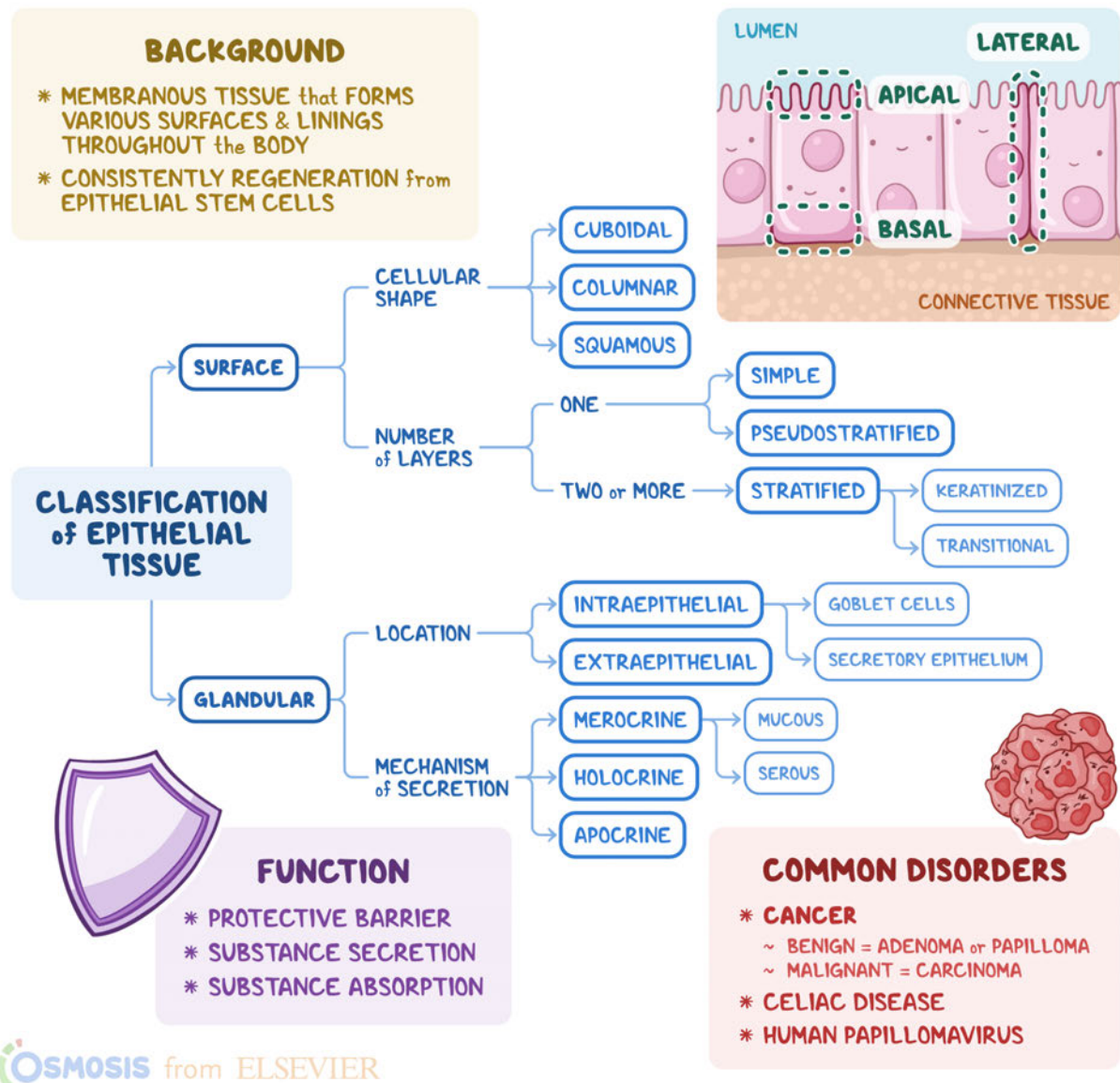


Figure 1.2. Schematical description of structure and functions of epithelia.
Reproduced from ¹⁴

1.2.2 Epithelial cell layer integrity and tight junctions

As illustrated in **Figure 1.3**, the integrity of the epithelial barriers is complex. It is maintained by various proteins between the cells, to ensure tight packing and limited portals of entry. Under normal circumstances, this prevents or regulates the passage of bacteria and other agents such as ions, water and solutes.¹⁵

The key elements in maintaining integrity are tight junctions (TJs).¹⁷ TJs are protein complexes composed of claudins, occludins, and junctional adhesion molecules (JAMs), attached to the cell's cytoskeleton through a scaffold-like structure composed of Zona Occludens (ZO) proteins (**Figure 1.3**).¹⁸

Situated at the apical pole of the epithelial cells, each of these TJ proteins play a central role. Claudins are very diverse (with over 20 different types identified in humans) and mainly regulate the permeability of TJs to various ions and solutes.¹⁹ The exact function of occludin is still unclear, but its interaction with other TJ proteins and the actin cytoskeleton is important for maintaining the barrier function. This is illustrated by its impairment observed when MDCK cells (Madin-Darby canine kidney cells) lack occludin.²⁰ Finally, JAMs are mainly involved in cell-cell adhesion and play a role in inflammation and vascular permeability.²¹

The dysfunction of TJs can lead to many issues. The first and most obvious consequence is the loss of the barrier function, allowing harmful substances and pathogens to infiltrate tissues and/or the bloodstream.¹⁷ Cell polarity, vital for the distinction between the apical and basolateral domains of epithelial cells, can also be disturbed, further affecting transport functions and signal transduction.²² Compromised TJs can also induce inappropriate immune responses, leading to inflammation, with many diseases (such as inflammatory bowel disease) linked to malfunctioning TJs.²³

Crucially, the function and importance of TJ varies with the type and location of the epithelium. For instance, in the pseudostratified columnar epithelia of the respiratory tract, the TJs are essential to ensure the exclusion of harmful particles and pathogens

and regulate air diffusion in the thin simple squamous epithelia found in alveoli.²⁴ Another example is the stratified squamous epithelia of the skin, which are thicker structures where TJs play a crucial role in maintaining a barrier against environmental threats.²⁵ As stated above, TJs are also key to regulating nutrient absorption and ion transport in the simple cuboidal and columnar epithelia (as found in the digestive tract and kidneys).²⁶

Finally, whilst the integrity and functionality of epithelial layers are heavily dependent on the integrity of tight junctions, other structures are also involved in maintaining this integrity. Below the TJs lay adherens junctions, composed of cadherins, directly linked to the actin cytoskeleton, providing another level of attachment between cells (**Figure 1.3**). There are also desmosomes, composed of desmogleins and desmocollins providing mechanical strength for the cell layer; gap junctions, enabling communication between cells by allowing the passage of ions and small molecules; and lastly hemidesmosomes, anchoring cells to the extracellular matrix.¹⁷

1.2.3 Adhesion of bacteria to epithelial cells

Following the access of various sites in the human host, both commensal (potential opportunistic pathogens) and obligate pathogens must find a way to establish themselves in the body, either by “simply” becoming part of a site-specific microbiota, or by adhering to the epithelial host cells.

Adhesion to epithelial cells requires bacterial surface proteins (generically named “adhesins”) to bind with cell surface receptors.²⁷ Adhesins are found on structures like

pili or fimbriae, which are hair-like organelles extending from the bacterial surface. These interact with specific receptors or carbohydrates on the surfaces of human cells in “a lock and key” mechanism, allowing bacteria to attach themselves firmly on surfaces and ensuring they can resist the natural flows and movements that might otherwise displace them.

Interestingly, whilst both commensal and obligate pathogen bacteria adhere to epithelial cells, the primary goal of commensal bacteria is often to establish a stable and non-inflammatory presence. They may use specific adhesins or surface structures to interact with host epithelial cells, but are generally less invasive and harmful.²⁸ Obligate pathogens, on the other hand, often have evolved more sophisticated mechanisms to interact with host epithelial cells, possessing adhesins and virulence factors allowing them to adhere tightly to host cells and initiate the infection process.²⁸

Some bacteria also interact with the extracellular matrix components that surround host tissues, through the recognition of components such as collagen, fibronectin, or laminin.²⁹ By binding to these structures, bacteria can both anchor themselves and penetrate deeper into the host's tissue in the case of some pathogens.²⁹

This initial attachment to either host cells or extracellular matrix is crucial for many pathogens, as it represents the preliminary step to establishing an infection. For example, certain strains of *Escherichia coli*, responsible for urinary tract infections, employ type 1 pili to attach to uroepithelial cells. This binding allows them to ascend the urinary tract, ultimately leading to infections in the bladder or even the kidneys.³⁰ In the gut, a classic example of a bacterium using adhesion to epithelial cells to

colonise them is enteropathogenic *Escherichia coli* (EPEC)³¹. After ingestion, EPEC interacts specifically with receptors on the surface of the host's intestinal epithelial cells through its adhesins and fimbriae on its surface (one well-known adhesin in EPEC is called intimin). This attachment is specific and allows the bacterium to adhere tightly to the host cells, inducing "attachment and effacement" lesions, which results in the intimate attachment of the bacterium to the epithelial cell surface. EPEC then forms pedestal-like structures beneath the adhered bacterium, helping the bacterium to maintain a stable attachment to the host cell while resisting the mechanical forces in the gut. This tight attachment and colonisation of the gut epithelium further allows EPEC to multiply and disrupt the normal functions of the host cells, leading to diarrhoea.³¹ A last example is *Helicobacter pylori*, a bacterium causing gastric ulcers by using a unique adhesin to bind to receptors on the gastric mucosa, allowing it to persist in the stomach's acidic environment.³²

1.2.4 Epithelial and mucosal immune systems

The immune system, operating as the first lines of defence against external bacteria is a critical element in this relationship between bacteria and epithelial cells by operating as the first lines of defence against external bacteria.³³ It will often recognize and attempt to remove any potentially harmful adhered bacteria. It can be divided into epithelial and mucosal immune response components, forming a sophisticated and collaborative network composed of various cells.

Epithelial cells play an essential role, beyond the previously described physical barrier: they actively participate in immune surveillance by secreting cytokines, such as

interleukins and tumour necrosis factor, which can recruit and activate immune cells to try and eliminate the bacteria.³⁴ Additionally, they can recognise pathogens via “pattern recognition receptors”, further enhancing the immune response.³⁵

When focussing on the gut epithelium, a few cells with a specific immune function are described:

- M cells capture antigens from the gut lumen and present them to underlying immune cells, serving as a bridge between the external environment and mucosal or systemic immune systems.³⁶
- Paneth cells, found at the base of the intestinal crypts, secrete antimicrobial peptides (directly targeting and killing pathogens) and maintain gut homeostasis by supporting the intestinal stem cells.³⁷
- Goblet cells produce mucus, which will both entrap pathogens and neutralise them with the IgA immunoglobulin it contains.³⁸

Beyond these specialised immune epithelial cells, the mucosal tissue contains classical immune cells, including dendritic cells (antigen-presenting cells) T and B lymphocytes (responsible for adaptive immune responses), natural killer cells and macrophages.^{39–41}

The diversity and abundance of various immune cells make the epithelial and mucosal immune system a dynamic and collaborative network, ensuring a rapid and effective response to any microbial challenge (including from commensal bacteria). Importantly, this immune system has also evolved to be tolerant to beneficial commensal bacteria.⁴²

Yet, it can be countered by many pathogens that have developed mechanisms to evade or suppress these immune responses.⁴³

1.2.5 Passage of bacteria through epithelium

As mentioned earlier, bacteria mainly cause infection by reaching sites where they are usually not present which are therefore vulnerable to their presence and toxic effects. Often, bacterial adhesion to epithelia is sufficient to trigger an infection. An example would be *Streptococcus pneumoniae*, often part of the healthy upper respiratory tract microbiota, that on reaching the lower respiratory tract causes disease following attachment to the alveolar epithelium.⁴⁴ Nonetheless, many bacteria seek to cross the epithelial barrier to reach other sites to exert their pathogenic power. The two main methods by which bacteria can traverse these epithelial barriers to further cause disease (either opportunistic or obligate pathogens) are the paracellular and transcellular pathways (illustrated in **Figure 1.4**).⁴⁵

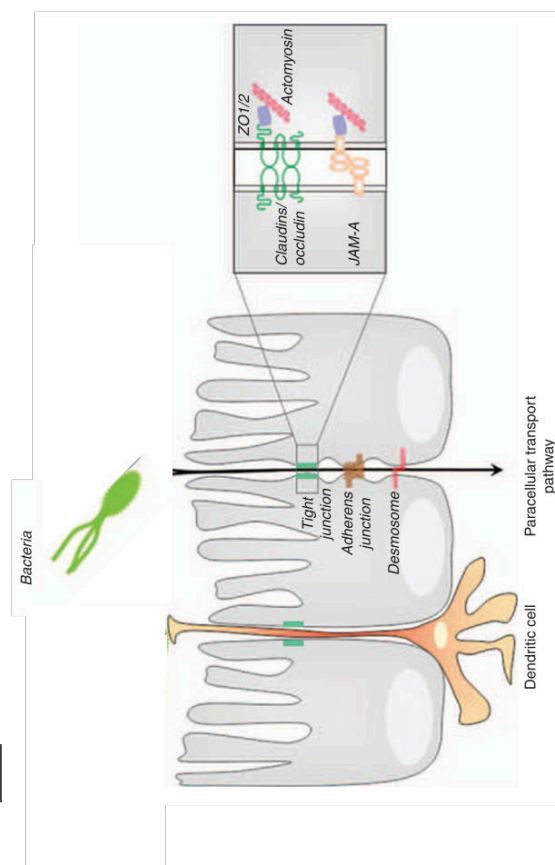
The paracellular route involves the passage between adjacent epithelial cells, therefore disrupting the TJs. Some bacteria have developed strategies that enable them to interact directly with TJs. In the gut for example, *Clostridium perfringens* produces an enterotoxin that will directly bind to claudin-4, causing its internalisation and intracytosolic destruction.^{16,46} Similarly, *Campylobacter jejuni* uses outer membrane vesicles to cleave intestinal epithelial cell E-cadherin and occludin.⁴⁷

The other mechanism used by bacteria for paracellular passage involves the use of the host cell's own signalling pathways to disrupt TJs, such as cGMP-dependent-protein, Rho GTPases, Myosin light chain kinases (MLCK) or cytokine secretion

cascades.⁴⁸ The activation or inhibition of these pathways will subsequently induce changes in the tight junction's protein expression or post-transcriptional events, modifying their structure and allowing bacteria to further pass through the resulting gaps that appear between cells. For example, enteropathogenic *Escherichia coli* secretes a heat-stable enterotoxin that will bind to the guanylate cyclase receptor of epithelial cells, forming cGMP.¹⁶ This then triggers the cGMP-dependent-protein kinase A pathway, dephosphorylating occludin, later redistributed away from the membrane to the cytoplasm.

The transcellular route involves bacterial passage directly through the epithelial cell. Bacteria employing this method usually bind to the epithelial cell surface via specialised proteins or receptors, initiating a series of intracellular events. One classic example is the invasion of intestinal epithelial cells by *Salmonella spp.*, utilising a specialised secretion system to inject effector proteins into the host cell, which then weakens the cell membrane, leading to bacterial engulfment. Once inside, the bacteria are often contained in vesicles, protecting them from the hostile intracellular environment and allowing them to propagate or move to adjacent cells.⁴⁹ These mechanisms are essential, because the passage of bacteria through epithelial barriers is not only a gateway to infection, but can also trigger an array of host responses. As detailed above, the host's immune system, upon detecting bacterial breach, can produce inflammatory cytokines, recruit immune cells, and initiate a cascade of events to counteract the invasion, leading to numerous clinical manifestations.⁵⁰

A



B

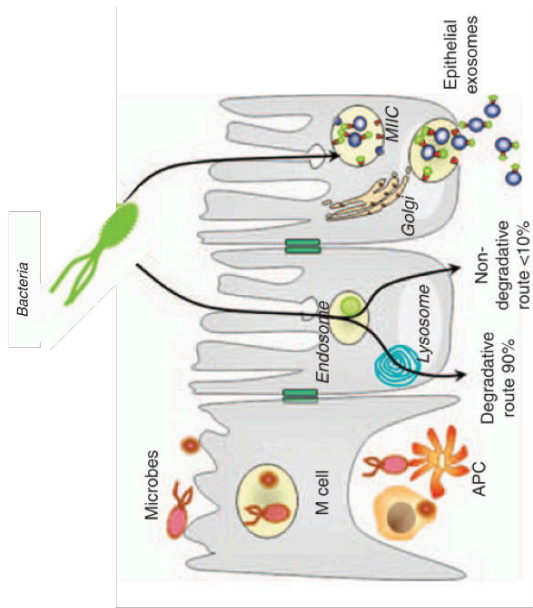


Figure 1.4. A. Representation of the paracellular pathway. B. Representation of the transcellular pathway. Adapted from ⁴⁵

1.3 Bacteria along the gut-liver axis

1.3.1 The gut-liver axis

1.3.1.1 *Anatomical description*

As illustrated in **Figure 1.5**, the gut-liver axis comprises the intestine and liver, interconnected by both the vascular portal system (gut to liver direction) and the biliary system (liver to gut direction).⁵¹

The gut itself comprises multiple layers, including the mucosa, submucosa, muscularis externa, and serosa. The mucosa contains epithelial cells and immune cells that absorb nutrients and prevent pathogens translocation. Once they crossed the intestinal barrier, these molecules reach the liver through the portal veinous system. The liver is divided into two main lobes, the larger right and smaller left, each composed of thousands of lobules. Lobules are the functional units of the liver and consist of hepatocytes arranged in rows around a central vein. Each lobule is also bordered by the portal triad, which consists of three key structures: a branch of the hepatic artery supplying oxygenated blood, a branch of the portal vein carrying nutrient-rich but deoxygenated blood from the gastrointestinal tract, and a bile duct collecting bile produced by hepatocytes. This structure is crucial as many molecules arrive from the intestine to the liver via the portal vein, are then processed or metabolised by the hepatocytes (which they join via the central vein), then excreted in the bile to return to the intestine, as further described below.⁵²

Liver

ECM modeling/Proliferation/Apoptosis

Hepatocytes

Central vein

HSC

EGFR

TLR-4

LPS

MadCAM-1

CCL-25

Portal vein

Bile duct

Hepatic artery

Gut Lumen

Gut microbiota

Gut epithelium

Lamina propria

Dysbiosis/Gut permeability

LPS

Blood vessel

PRR

Gut-primed T-cell

$\alpha\beta 7$

CCR9

MadCAM-1

CCL-25

19

Role of the gut-liver axis

The gut-liver axis has been described in a recent review as “the bidirectional relationship between the gut, its microbiota, and the liver, arising as a result of interactions between signals generated by dietary, genetic and environmental factors”.⁵³ This relationship is mediated through a variety of factors, including the circulation of bile acid, nutrients, metabolites (some produced by the gut microbiota) and, importantly, bacterial pathogens and/or their endotoxins.

Bile acids are produced by the liver, stored in the gallbladder and released into the small intestine after a meal to aid in the digestion and absorption of fats. The composition of bile acids in the intestine can influence the composition of the gut microbiota. Conversely, changes in gut microbiota can affect the bile acid metabolism in the liver, potentially influencing liver diseases (see below).⁵⁴

In the other direction (from the gut to the liver), the most crucial structure is the barrier formed by, respectively, the microbiota, the intestinal epithelium, immune cells in the submucosal space and the vascular endothelium of the portal system (see **Figure 1.5**). Its function is to selectively let nutrients and metabolites through to the portal circulation, while preventing the translocation of potentially harmful elements (such as bacteria or their toxins). The gut microbiota not only physically prevents other harmful bacteria from reaching the epithelial layer, but also plays a crucial role in metabolising dietary components into various metabolites. For instance, short-chain fatty acids like acetate, propionate and butyrate are produced through the fermentation of dietary fibres by gut bacteria. These have been shown to have beneficial effects on liver health

by modulating lipid metabolism, reducing inflammation and improving insulin sensitivity.⁵⁵

As described above, the gut epithelial cell barrier, underlying the microbiota, is a physical, electrical (carrying a negative charge) and immunological barrier (secreting antimicrobial peptides) against pathogens. In the context of bacteria, along with the portal vascular endothelium, the gut epithelium prevents the translocation of bacteria and/or their products, such as lipopolysaccharide (LPS), from entering the portal bloodstream and reaching the liver.^{52,56}

1.3.2 Pathophysiological implications

1.3.2.1 *Role of the gut microbiota in liver diseases*

The human gut hosts trillions of microbial inhabitants, collectively named as the gut microbiota. This community consists predominantly of bacteria, along with other microorganisms such as viruses, fungi, and protozoa. These organisms play an essential role in the digestion of food, synthesis of vital compounds, modulation of the immune system and protection against harmful pathogens.⁵⁷ Notably, alterations in the gut microbiota composition, or dysbiosis, have been associated with various liver diseases, including alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), cirrhosis, and hepatocellular carcinoma. For instance, chronic alcohol consumption can lead to changes in gut microbiota composition and increased intestinal permeability, resulting in the translocation of bacterial products like LPS (Lipopolysaccharide, located in the outer membrane of Gram-negative bacteria, responsible for its pathogenicity) to the liver. This can stimulate inflammatory

responses and contribute to the progression of ALD.⁵⁸ Another example is the gut dysbiosis often present in patients with cirrhosis, which can contribute to complications like hepatic encephalopathy, a condition characterized by cognitive impairments due to liver dysfunction. The mechanism may involve the production of neuroactive compounds by the altered microbiota.⁵⁹

1.3.2.2 *Gut commensal and opportunistic bacteria*

Among the commensal bacteria, the microbiota can also host opportunistic pathogens, generally harmless in a healthy individual, but causing disease if the host's defence mechanisms are compromised.

Clostridioides difficile is a classic example of such opportunistic pathogens in the gut.⁶⁰ Under normal circumstances, the proportion and absolute number of *C. difficile* is controlled by the competing microbial community. However, administration of broad-spectrum antibiotics will eradicate most of the gut bacteria, leaving room for *C. difficile* to proliferate, leading to infections that can cause severe diarrhoea and inflammation of the colon.⁶⁰ The toxins produced by this bacterium are also key in its pathogenesis, damaging the epithelial cells and inducing an inflammatory response.

Enterococci, mostly *Enterococcus faecalis* and *Enterococcus faecium*, are another example. They are usually considered as harmless residents of the gastrointestinal tract, but they have recently emerged as significant opportunistic pathogens causing infections in extraintestinal sites, particularly the urinary tract or intravascular devices. This is probably a result of their *in vivo* ability to colonise and translocate through intestinal cells layers, using various virulence factors as aggregatory substances, specific antigens (providing survival in phagosomes) and gelatinases.^{61,62}

To summarise, the gut microbiota is a delicately balanced ecosystem, where bacteria play both protective and potentially harmful roles. With the rising prevalence of antibiotic-resistant strains, it becomes even more essential to study these opportunistic bacteria, as they pose an imminent threat in the evolving landscape of global health.

1.3.2.3 *Bacterial infections of the liver*

Bacterial infections affecting the liver can present with three different outcomes: hepatitis, granulomatous diseases and abscesses.

Hepatitis strictly refers to inflammation of the liver and is mostly caused by viral infection (Hepatitis A, B, C, D or E viruses) or by external toxicity (alcohol, drugs).⁶³

Bacterial infections leading to hepatitis are fairly rare, but have been described for *Bartonella henselae*, *Brucella spp.*, *Coxiella burnetii*, *Leptospira interrogans*, *Yersinia pestis* and *Treponema pallidum*.⁶⁴

Granulomatous liver disease consists of the development of granuloma, an organised collection composed of macrophages and fibroblasts, smaller than an abscess and typically developing over a long period of time. Its main bacterial causes are *Mycobacterium tuberculosis*, but *Brucella spp.* and *Coxiella burnetii* are alternative potential causes.^{65–67}

The principal and most critical bacterial infections in the context of the liver are pyogenic liver abscesses (PLA), consisting of pus (i.e. proteins and dead polymorphonuclear neutrophils) forming either as solitary or multiple masses in the liver parenchyma. Bacteria reach the liver through different routes, the two main ones

both making a clear use of the gut-liver axis described above: (1) the most common route is ascension from the biliary tract (often associated with biliary tract obstruction (gallstone, neoplasia, etc))⁶⁸, but (2) the leakage of bacteria into the portal bloodstream from gut colonisation also represents a major route for bacteria to reach the liver. Other routes include direct extension from intra-abdominal infections (e.g. peritonitis) or septic embolisation via the hepatic artery during bacteraemia.⁶⁹

PLA can cause both liver-specific, localised symptoms and signs (abdominal pain and discomfort, liver enzymes elevation involved in cytolysis and cholestasis) and severe systemic inflammation and sepsis signs (fever, tachycardia, low blood pressure, elevated inflammatory markers) potentially leading to septic shock and death.⁷⁰ Although moderately common in the UK (with an annual incidence of 2.3 per 100,000 population⁷¹), their mortality is estimated to be as high as 12%, which makes them a serious healthcare challenge.⁷¹

The microbiological characteristics of these abscesses reveal that the main causative pathogen is *Klebsiella pneumoniae* (*Kp*), retrieved in almost half of the cases in recent studies, just ahead of *E. coli*, anaerobes and *Enterococcus* spp.^{70,72}

Interestingly, these *Kp*-associated PLA can be caused by two types of *Kp*: (1) the classical opportunistic *Kp*, ascending the biliary tract from the gut following defects in the bile flow defence mechanism (e.g. in case of obstructed bile duct by lithiasis, neoplasms or infections). These are most commonly polymicrobial and form larger abscesses compared to (2) Hypervirulent *Kp*, causing abscesses in patients without

biliary tract disorders, therefore thought to reach the liver from the gut through the portal system.^{73–76}

1.4 Classical *Klebsiella pneumoniae*, a common opportunistic pathogen

Klebsiella pneumoniae is an aerobic rod-shaped Gram-negative encapsulated bacterium belonging to the Enterobacteriaceae family, present in the environment and commensal in animals and plants.^{77,78} It is a highly adaptable bacterium that has evolved into various lineages, mainly due to horizontal gene transfer. Each of these lineages has a distinct virulence and resistance profile.⁷⁹ Certain lineages, notably the sequence-type (ST) 258, 15 and 20 clones, have been associated with multidrug resistance and global hospital outbreaks;^{80,81} others, like ST23, 65 or 86, have been associated with the hypervirulent phenotype (see below).^{82,83} The *Klebsiella* genus also comprises other species, like *Klebsiella variicola*, *Klebsiella oxytoca* and *Klebsiella quasipneumoniae*, which have been occasionally linked to human infections, but are more often found in environmental samples, such as water and soils.⁸⁴

Kp are associated with human infections exacerbated by increasing levels of antibiotic resistance. They are known to colonise the nasopharynx, but first and foremost the digestive tract, with up to 6% of the general population excreting *Kp* in their stools.^{85,86} This rate is more significant in hospital settings, where its gut carriage is exacerbated by antibiotic use and found in around 20% of all hospitalised patients^{85,87} and up to 77% in intensive-care units.⁸⁸ Interestingly, the presence of *Kp* in the gastrointestinal microbiota acts as the main source of hospital-acquired infection (HAI) where 5% of the colonised patients will develop an infection during their hospitalisation, caused by their own strain in 80% of the cases.⁸⁹

Classical *Kp* (*cKp*) is therefore considered as an opportunistic pathogen, causing hepatobiliary but also urinary, pulmonary or blood stream HAI (hospital-acquired infections) in comorbid or immunocompromised patients.^{88,90,91}

Concerningly, *Kp* represents the third and fourth most common pathogen causing HAIs in the United States (9.9% of the cases)⁹² and in Europe according to a study conducted by the European Centre for Disease Prevention and Control (6.8% of 10076 microorganisms isolated).⁹³

1.5 Hypervirulent *K.pneumoniae*

1.5.1 Background

Since the 1980s, strains of *Kp* causing community-acquired invasive and aggressive tissue infection in otherwise young and healthy individuals have been described and designated as Hypervirulent *K.pneumoniae* (Hv*Kp*).

Hv*Kp*'s main clinical manifestation is a syndrome called community-acquired primary invasive liver abscess, which comprises of pyogenic liver abscess (Hv*Kp*-PLA) with potential dissemination to other sites such as the lungs (pneumonia), eyes (endophthalmitis), CNS (central nervous system, i.e brain abscesses) and soft tissues.^{94,95} This is potentially posing a significant route to liver damage, loss of liver function and septic shock.

The name “hypervirulent” comes precisely from the fact that Hv*Kp*-PLA mostly occur in young individuals with few pre-existing conditions, as the only independent risk factors identified in the host so far are diabetes and non-alcoholic fatty liver disease.^{70,96–99} Hv*Kp* therefore don't need a defect in host's immunity to cause the disease, behaving like obligate pathogens, as opposed to classical opportunistic *Kp*.¹⁰⁰

Initially detected in Asian countries (Taiwan, South Korea, China, Singapore), where its prevalence is still the highest, it is increasing globally, with more and more sporadic cases reported in the United States and in Europe.^{101–104} Since the characterisation of this syndrome, it has been shown that Hv*Kp* accounts for more than 80% of PLA cases in South East Asia and up to 9% of all cases worldwide.¹⁰⁵

In terms of prognosis, despite affecting mostly non-comorbid patients, Hv*Kp*-PLA mortality rate is estimated as high as 11%, relative to *cKp*-PLA.⁹⁶ Interestingly, phylogenetic studies, using both clinical and genetic characteristics, have clearly identified some *Kp* clones sharing a common ancestor associated with this hypervirulent phenotype (**Figure 1.6**), i.e. sequence-type (ST) 23, 25, 65, 66, and 86.

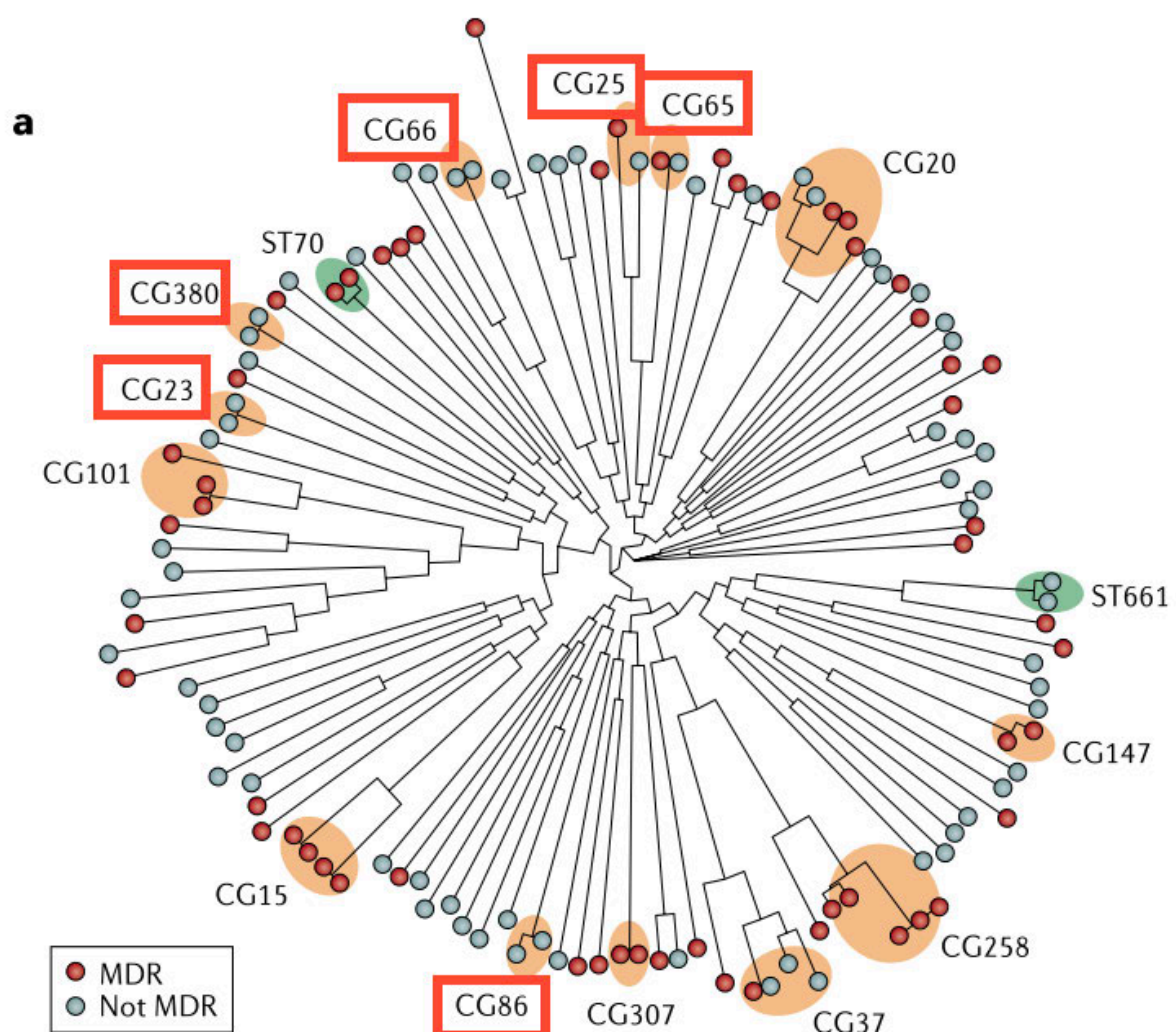


Figure 1.6. Phylogenetic tree of the main clonal group of *Kp*, with the ones associated with Hv*Kp* framed in red. Adapted from ¹⁰⁶.

Recent in-depth genomic analyses of *HvKp* isolates showed that strain SGH10, a member of the ST23 (responsible for more than 80% of *HvKp*-PLA cases) is an archetypal representative of *HvKp* causing PLA.¹⁰⁷ SGH10 was isolated in Singapore in 2014 from a 35-year-old liver abscess patient with no diabetes mellitus or other comorbidity.¹⁰⁸ Harbours the capsule serotype K1, this strain has been designated so because, as illustrated in **Figure 1.7**, (1) its genome is part of the predominant sublineage of CG23 (CG23 – I) and (2) it possesses all the *HvKp* virulence factors known to date: a virulence plasmid (carrying *iuc*, *iro*, *rmpA* and *rmpA2* genes, encoding the siderophores aerobactin, salmochelin, hypercapsule and HMV phenotype) and ICE*Kp10*, an Integrative and Conjugative Element (chromosomally integrated mobile genetic element) carrying yersiniabactin (*ybt*) and colibactin loci (*clb* or *pks*).

1.5.2 Delineation of HvKp and cKp

The distinction between *cKp* and *HvKp* is not clear-cut. It was initially based on clinical criteria, the two main clinical differences being that *HvKp*-associated PLA has the potential to metastasise and develop without existing anterior biliary tract disease (present in more than 60% of *cKp*-PLA cases).¹⁰⁹

However, this clinical distinction faces two main hurdles: (1) In addition to the differences mentioned above, *cKp* and *HvKp*- PLA mainly share the same clinical spectrum (fever, abdominal pain, elevated liver enzymes, inflammatory syndrome), making them hard to distinguish. (2) *HvKp* infections can occur patients who are immunocompromised hospitalised patients.

The second method proposed to distinguish *HvKp* from *cKp* is based on their microbiological phenotypical properties (see **Figure 1.8**). As developed in detail below, *HvKp*'s main microbiological characteristics are its thick capsule and hypermucoviscous phenotype, which appears as colonies producing a > 5mm string of mucosal extension when stretched from an agar plate. However, some strains are capable of causing *HvKp*-like infections, but are not hypermucoviscous, and some studies have reported up to 20% of *cKp* presenting a positive string test.¹¹⁰

Also, *HvKp* has been shown to secrete significantly more siderophores (iron-scavenging molecules) than *cKp*, mainly consisting of aerobactin^{111–113}, but no clear link with human infections has been made yet.

Finally, many *HvKp* strains secrete the genotoxin colibactin, which has been linked to virulence in mice models.¹¹⁴ However, some *cKp* secrete it and some *HvKp* don't,

making its precise contribution to the hypervirulent phenotype unclear. The latest and most in-depth way of differentiating HvKp from cKp is based on their genotypic features, as HvKp harbours some genes coding for specific virulence factors (see below). In that regard, Russo et al. have recently identified genes coding for siderophores (*iuc*, coding for aerobactin) and hypermucoviscosity (*rmpA(2)*) as strongly associated with HvKp.¹¹⁰ Yet, some strains within the CG23 carry both genes but do not express typical HvKp phenotypes, as these probably result from a more complex regulation network.

Taken together, a precise distinction of what is HvKp relative to cKp has yet to be agreed on. The most accurate way of defining a strain as HvKp is probably the combination of these clinical, phenotypic and genotypic factors, although there will always be a grey area.

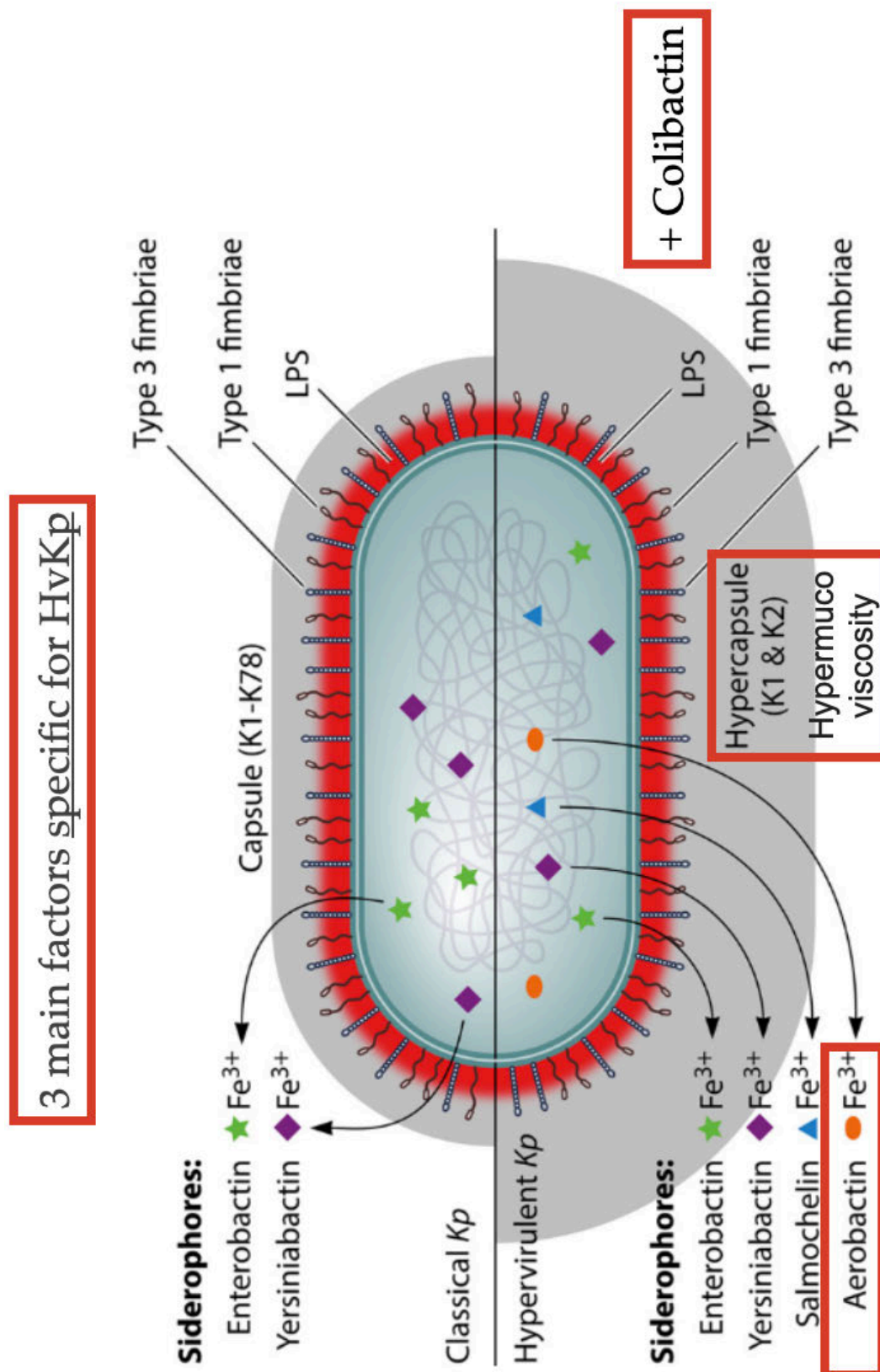


Figure 1.8. Main microbiological factors delimitating HvKp from cKp. Adapted from ¹¹⁵.

1.5.3 Kp-ubiquitous virulence factors

All *Kp* strains, including *cKp*, have genes encoding “general” virulence factors, which explains its overall pathogenicity. These genes are encoded by the core genome (defined as genes present in more than 95% of all strains population, 2000 genes in *Kp*)^{79,116}. This is opposed to the accessory genome, i.e. genes present only in certain strains of *Kp*, varying between isolates and representing up to 4000 genes. Most of the genes responsible for *HvKp*’s virulence are on the accessory genome and can be highly mobile and transferable through either plasmids or chromosomal integrative and conjugative elements (ICE).¹¹⁷

The virulence factors encoded by the core genome and therefore present in all *Kp* strains are the capsule, the siderophore enterobactin, the LPS and the fimbriae.

The capsule is encoded by the capsular polysaccharide synthesis (*cps*) locus. It is composed of exopolysaccharides attached to the outer membrane (OM) of the bacterium, forming a slimy layer that surrounds the bacterial cell. It mainly contributes to *Kp* virulence by evading the host’s innate immunity, both by the physical barrier it provides, but also by hiding bacterial surface antigens.^{115,118} It also possesses extracellular properties linked to thwarting binding the macrophage mannose receptor and utilising molecular mimicry of host-factors.^{119–121} The capsule also participates to biofilm formation, along with type-3 fimbriae (see below).

Enterobactin belongs to the family of high-affinity iron acquisition “siderophores”, molecules scavenging surrounding iron necessary for in vivo infection. This function is

crucial for bacterial growth and replication, especially in iron-depleted conditions (typically in the human host).¹²² *In vivo*, enterobactin has been shown to promote *Kp*'s colonisation and dissemination from the lungs.¹²³ Whilst it has the highest iron affinity of all siderophores, enterobactin is also the best recognised by the human immune system, precisely because of its ubiquity.¹²⁴ It is neutralised by Lipocalin-2, an antimicrobial peptide secreted by innate immunity cells. This doesn't kill *Kp* but stops its growth by preventing the bacteria from exploiting the surrounding iron.^{125–127}

LPS is a lipid complex found on the OM of *Kp*, where it acts as an endotoxin. It is composed of an O antigen, a core oligosaccharide and lipid A. It has a dual role, hiding certain *Kp* antigens from the humoral immune system, but also being extremely immunogenic and therefore potentiating septic shock, mainly through the innate immune system activation.^{128,129} This immune activation is induced by lipid A binding the TLR4 (Toll-Like Receptor) of macrophages, which in turn secrete cytokines, leading to further macrophage and neutrophil recruitment.¹³⁰ The virulence provided by LPS has been demonstrated by showing attenuated pathogenicity of lipid A mutant strains in a mice respiratory infection model.¹³¹ Interestingly, one of the mechanisms by which the capsule evades the immune response is by hiding this lipid A from the TLR4 receptors on macrophages.^{132,133} Some *Kp* can also stay protected from immune response by modifying their lipid A structure.¹³⁴

Fimbriae or pili are filaments, also attached to the OM, and act as adhesive structures (belonging to the family of "adhesins"), mainly to epithelial cells.¹³⁵ These fimbriae can be type I, which are thin protrusions of the OM, mostly responsible for bacterial adhesion on human epithelial or mucosal layers, or type III, which are helix-shaped,

also enhancing adhesion to epithelium but more importantly participating in biofilm formation.¹³⁶ This last is a matrix created by the strong adhesion of bacteria to a surface (mucosae or foreign material) and to each other, forming communities and protecting them from antibiotics or the immune system. Interestingly, despite the *in vitro* demonstration of increased adhesion to epithelial cells by type I and type III fimbriae, they don't seem to play a role in gut and lung colonisation.¹³⁷ The main contribution of these fimbriae to *Kp*'s pathogenicity is their ability to bind to abiotic surfaces such as catheters or other implantable devices.^{138,139}

1.5.4 HvKp-specific virulence factors

As illustrated in **Figure 1.9**, the hypervirulent character of HvKp arises from the fact that it possesses several accessory genes (present either on a plasmid (“virulence plasmid”) or integrated in the genome), increasing the expression of the capsule and the siderophores, but also coding for exogenous toxins (e.g. colibactin). All these factors appear to be necessary to confer hypervirulence, and none seems sufficient on its own.¹⁰⁵

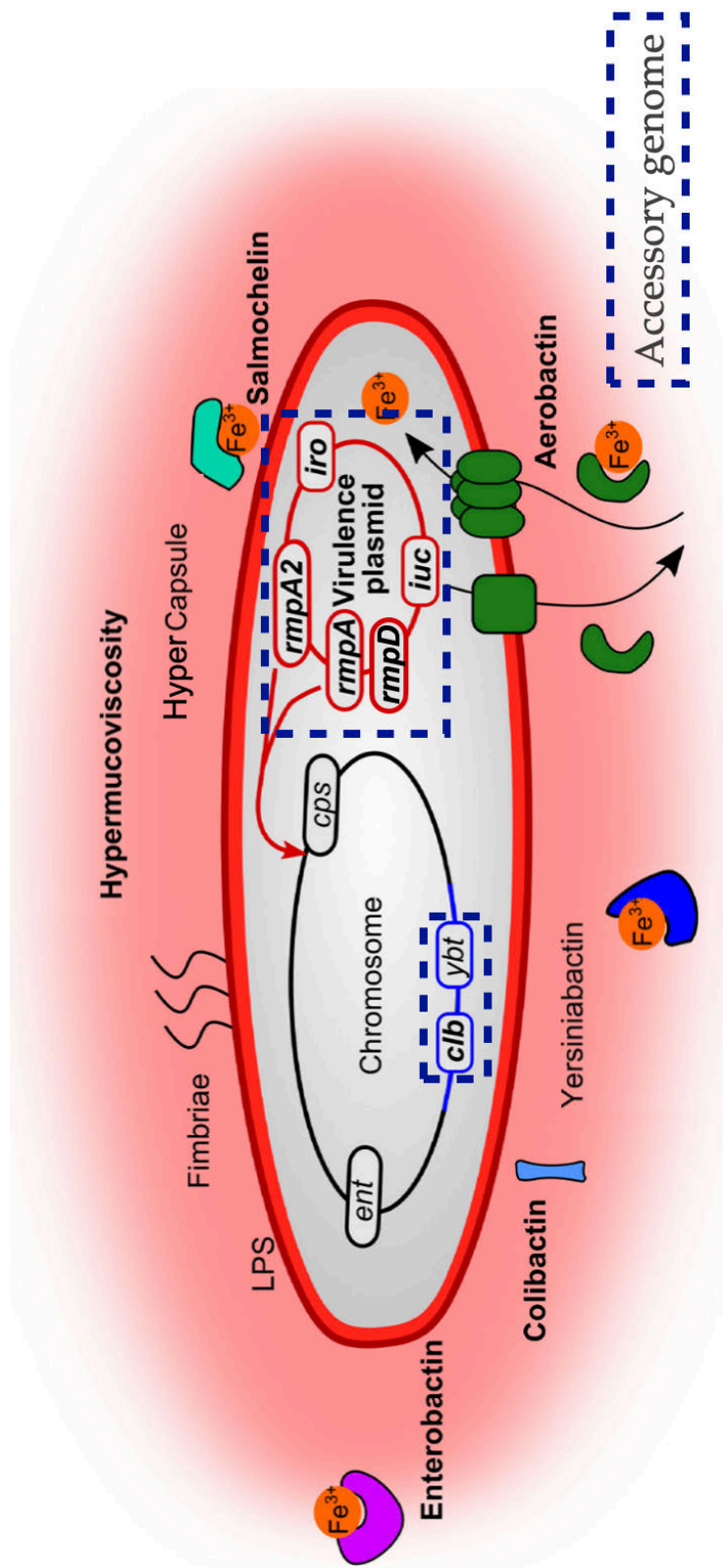


Figure 1.9. Schematic representation of SGH10, with its virulence factors and the genes encoding them. Adapted from ⁹⁵.

1.5.4.1 Capsule – Hypermucoviscosity

The capsule structure and hypermucoviscous phenotype (HMV) are the main features of *HvKp*; they are distinct but interlinked elements.

- Capsule

The capsule is structurally present in all *Kp* strains, but the differences in its properties influence the bacterial pathogenicity. It is coded by the *cps* cluster, comprising over 20 genes and controlled by the promoters *galF*, *wzi* and *manC*.

The main feature of *HvKp* is the thick capsule (“hypercapsule”) it presents. **Figure 1.10** shows the mechanism of this overproduction, that results from the activity of several genes positively regulating transcription of the *cps* locus. These include *rmpA* and *rmpA2* (“regulator of mucoid phenotype”) genes, located on the virulence plasmid and acting as positive regulators, mostly of *manC* via the activation of *rmpC* and *galF* via the activation of RcsA/B (two-component signal transduction system regulation of capsule synthesis, originally identified in *E. coli*, probably sensing the environment and increasing capsule production accordingly).¹⁴⁰ *rmpA* is itself upregulated by the recently described *kvrA* & *kvrB* (*Klebsiella virulence regulators*), which have the MarR-family domains, found in the chromosome of both *cKp* and *HvKp*, but regulating capsule production only in *HvKp*.^{141,142}

This thick capsule potentiates resistance to phagocytosis and intracellular killing by macrophages, but also resistance to complement.^{94,120,143–146} A recent study by Tan et al. identified the capsule as crucial for dissemination of *HvKp* to distant organs, but not for its persistence in the gut.¹⁴⁷ This was demonstrated by generating mutants lacking capsule ($\Delta wcaJ$ and $\Delta rmpA$) and comparing them to the wild type strain in an *in vivo*

murine infection model, performing both a competitive assay in the gut and an intraperitoneal injection. Interestingly, these mutants were shown as fitter than wild type strains in the competitive assay, but disseminated less well when injected intraperitoneally to the mouse.¹⁴⁷ This has however been contradicted by another study using a murine model to look at the colonisation and persistence of HvKp in the gut, showing that capsule is necessary for both of these processes.¹⁴⁸

Finally, HvKp capsular serotype also seems to be related to virulence, with HvKp strains mostly expressing the K1 or K2 serotype, although no clear explanation as to how these serotypes could contribute to hypervirulence has been proposed to date.¹⁴⁹

- Hypermucoviscosity

HMV, on the other hand, represents the viscidness of the bacteria. By generating mutants lacking the hypercapsule but retaining HMV phenotype, Walker et al. showed that HMV is a distinct phenotypic functionality of the bacteria and not a mere consequence of hypercapsule.¹⁵⁰

Recently, a gene independently contributing to HMV has been identified and designated as *rmpD*. It consists of a short 96bp gene neighbouring *rmpA(2)* on the virulence plasmid, coding for a 58 amino acid protein.¹⁵⁰ It has also been recently demonstrated that HMV was not linked to any biochemical modification of the capsule, but rather to a regulation of the capsule's chain-length distribution through the interaction of RmpD with *wzc*, a tyrosine kinase protein involved in capsule export from the inner to the outer membrane.¹⁵¹

HMV contributes to the virulence of *HvKp* by reducing adherence/association with host cells, leading to both avoiding phagocytosis and paradoxically improving persistence in the mouse gut.^{147,150,152}

Despite an increasing number of studies, the precise mechanism which results in the HMV phenotype and *in vivo* effects in pathogenicity and liver tropism is still debated. It has however fostered increasing interest over recent years, while its role is probably underestimated and confused with the capsule function, as some effects attributed to the loss of the capsule might actually be the consequence of the subsequent loss of HMV.¹⁴⁷

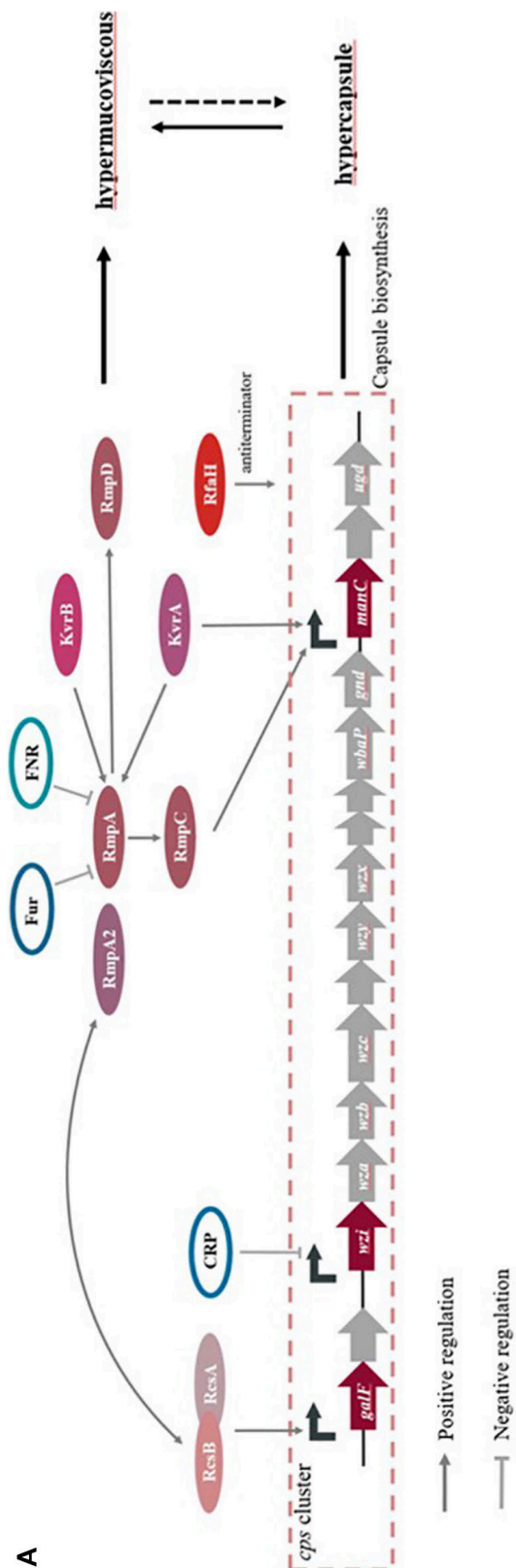


Figure 1.10. Mechanism of the genes involved in the regulation of the *HvKp* capsule and hypermucoviscosity levels. Reproduced from ¹⁵³.

1.5.4.2 Siderophores

Siderophores seem to be another essential element in the pathogenesis of HvKp. Classical *Kp* only expresses *enterobactin* (encoded by *ent*, on the core genome), recognised by the innate immune system, making it poorly efficient for bacterial pathogenicity.¹⁵⁴ HvKp secretes an additional three siderophores relative to cKp: *aerobactin* (*iuc*), *yersiniabactin* (*ybt*) and *salmochelin* (*iro*), all encoded by the accessory genome.^{155,156}

So far, only aerobactin has been shown to increase the virulence *in vivo*: it represents more than 90% of total HvKp siderophore excess production and is the only one enhancing both bacterial survival in human fluids (ascites and serum) and virulence in mouse systemic and pulmonary infection models.^{156,157} It is however unclear if aerobactin or siderophores in general contribute to the formation of liver abscesses, since its precise roles in both dissemination and liver tropism of HvKp haven't been established.

1.5.4.3 Colibactin

The genotoxin colibactin has been initially described in *E. coli*, where it is a secreted product of 19 genes located on the *pks* (or *clb*) gene cluster.¹⁵⁸ It causes alkylation and intercalation of DNA, leading to double-strand breaks.^{159–161} It has recently been found in *Kp*, within the same genomic island, acquired and integrated to the genome via ICEKp10 (Integrative and Conjugative Element). Initially described in around 20% of all the *Kp* strains in Taiwan¹⁶², it has later been shown that almost 80% of HvKp K1 strains carry *pks*.¹⁶³ In *Kp*, a mouse model has suggested that colibactin facilitates gut

colonisation and subsequent translocation to the portal circulation, further causing liver damage.¹⁶⁴

1.5.5 HvKp and antimicrobial resistance

Kp is one of the most critical bacteria in terms of acquiring antimicrobial resistance, an increasing and major threat for humanity.¹⁶⁵ It is part of the ESKAPE group of pathogens (which also includes *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), defined as pathogens “representing a formidable challenge in the treatment of nosocomial infections”.^{166,167} The World Health Organization has recently listed ESKAPE pathogens in the list of 12 bacteria against which new antibiotics are urgently needed.¹⁶⁸

Antimicrobial resistance in HvKp arises from various mechanisms, including the production of extended-spectrum beta-lactamases and carbapenemases, which undermine beta-lactams. These are often carried on plasmid and therefore horizontally transmitted between strains. Interestingly, most of the HvKp strains described to date are sensitive to antibiotics, making HvKp-PLA readily curable with first or second-line antibiotics (e.g. amoxycillin-clavulanate, ceftriaxone or quinolones) if treated early.⁹⁵

However, recent studies show molecular convergence events between antimicrobial resistance and hypervirulence, i.e. the horizontal transfer of either resistance mechanisms to last-line antibiotics (e.g. plasmid-mediated carbapenemases) by HvKp or virulence plasmids by multidrug-resistant (MDR) cKp strains.^{169–171} The resulting bacteria, designated as “superbugs”, can lead to severe and hard to treat infections, often fatal, and therefore represent a serious public health threat.¹⁷² The first sporadic

cases have been described in the early 2010s in Asia, but cases have now been reported worldwide (**Figure 1.11**).

Recent genomic evolutionary analyses of MDR *Kp* strains show significantly higher gene acquisition (chromosomal recombination and plasmid diversity) than Hv*Kp*, suggesting that MDR strains are more likely to acquire Hv genes.¹⁷³ For instance, five patients were reported to be fatally infected from lower respiratory tract infection caused by MDR-Hv*Kp* in 2016 in Hangzhou, China. These strains were originally carrying *bla*_{Kpc-2} and became hypervirulent after the acquisition of the pLVPK virulence plasmid.¹⁷⁴ Reassuringly, these infections were acquired in hospital and not in the community, in line with the opportunistic profile of c*Kp*. MDR-Hv*Kp* have recently been detected in the UK as well: a study conducted by Public Health England in 2018 found a K1 CG23 strain carrying *bla*_{OXA48} and a *bla*_{NDM-1} *Kp* strain presenting with the pLVPK virulence plasmid.¹⁷⁵

If the clinical significance of these isolates was not specified, another K1 CG23 harbouring a *bla*_{NDM-1} carbapenemase gene was described in London, in a 63-year-old patient who died after 7 days of hospitalisation.¹⁷⁶

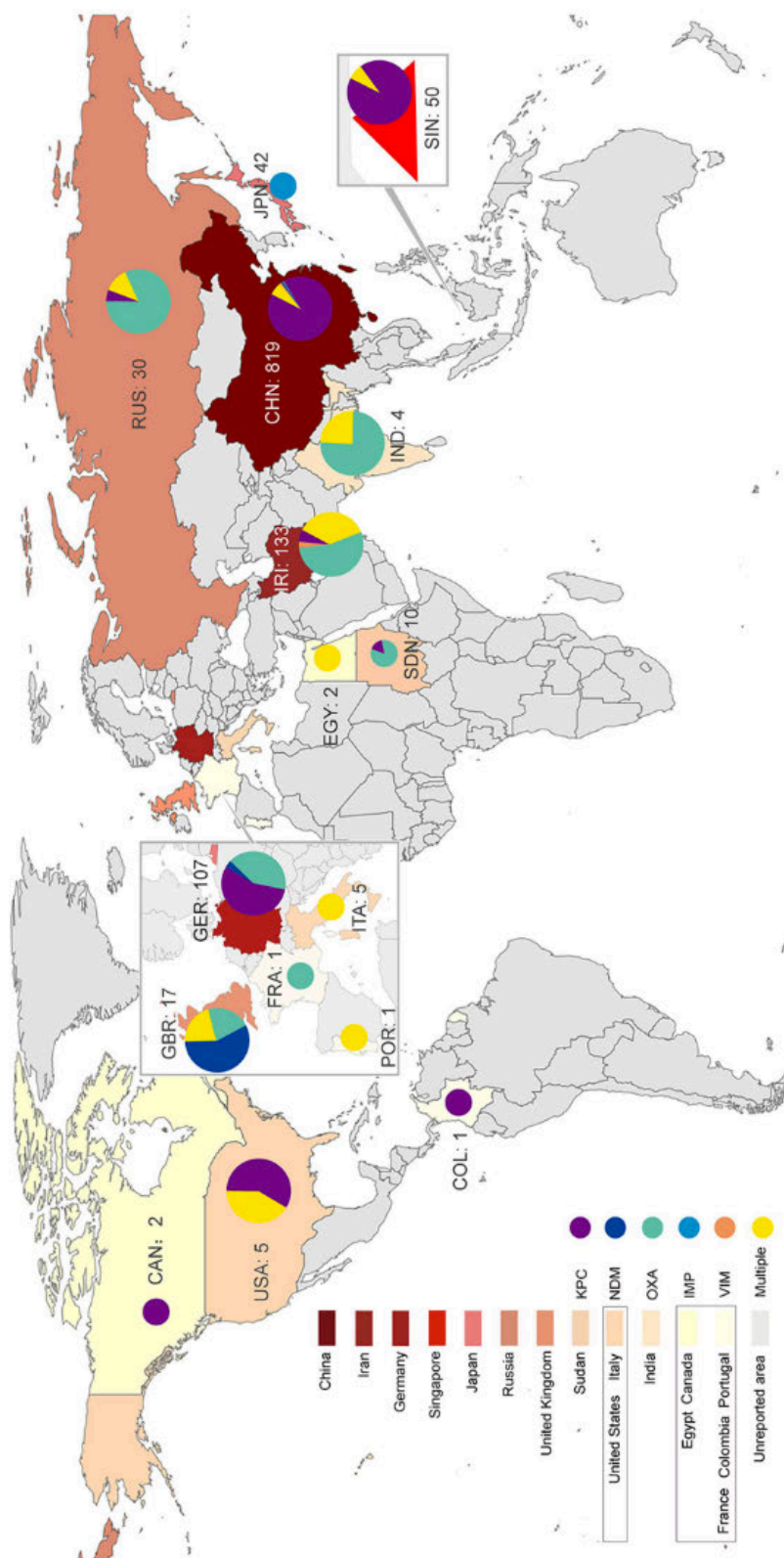


Figure 1.11. Distribution of Carbapenamases producing-HvKp in the world, with their main mechanism of resistance. Reproduced from ¹⁷⁷.

Yet, beyond this AMR and in addition to its own microbiological virulence characteristics, one of the vital features of Hv*Kp* is its avoidance of the immune system.

1.5.6 (Hv)*Kp* and the immune system

As summarised in **Figure 1.12**, various *in vitro* and mice models have clearly identified that neutrophils, macrophages, NK and T lymphocytes, epithelial and dendritic cells were essential defence actors against *Kp*. Along with the phagocytosis function carried out by the neutrophils and macrophages, all these cells will secrete TNF α , CXCL15, IL-6 and IL-8, but also activate the IL-23/IL17 and IL-12/IFN γ axes, further leading to *Kp*'s engulfment and killing.^{178–182} The complement also plays a major role, opsonising or directly killing *Kp*.¹⁸³

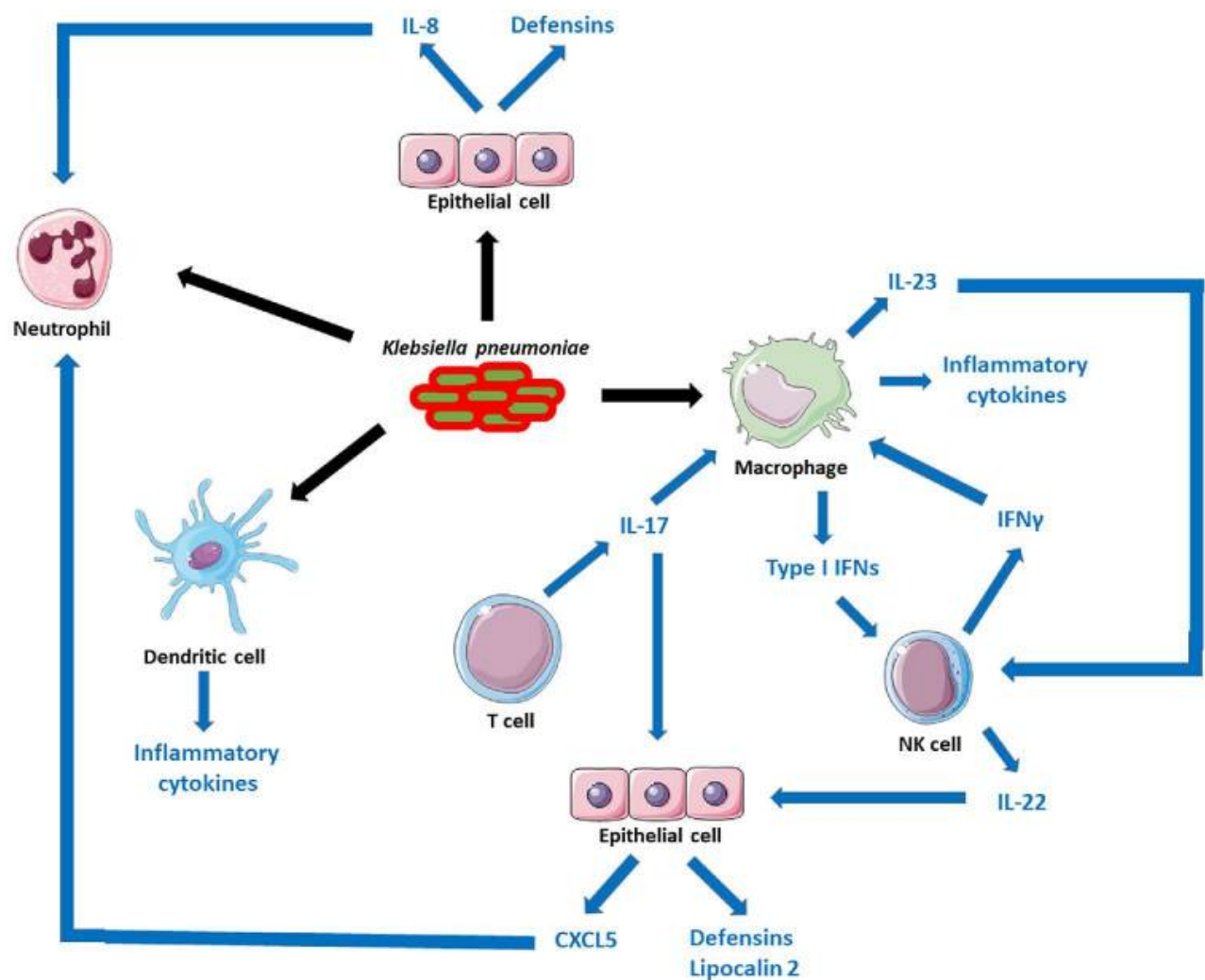


Figure 1.12. Summary of innate immunity mechanisms to try and contain *Kp* infection. Reproduced from ¹⁸⁴.

In return, *Kp* has adapted and developed many mechanisms to mitigate this immune activation, thereby promoting its pathogenicity.

First and foremost, *Kp*'s capsule is a paramount feature, allowing it to hide its surface molecules, normally recognised by the cells or soluble effectors of the immune system. It has been shown as protecting against phagocytosis, but also against the

complement, and decreasing the secretion of proinflammatory cytokines like TNF (Tumour Necrosis Factor)- α and IL (Interleukin)-6.^{185–188}

As described above, the immune evasion is one of the main characteristics of *HvKp*, contributing to its ability to infect immunocompetent hosts. Crucially, this is probably due, at least in part, to its thick, viscous capsule, which potentiates and diversifies the immune-evasive features described for all *Kp*. For instance, a *HvKp* strain (*Kp* 52145) has recently been shown as twisting the mouse alveolar macrophages function from M1 (pro-inflammatory) to M2 (anti-inflammatory), using its capsule antigens to activate the type I IFN-IL10-STAT6 axis via TLR-2 and 4 receptors.¹⁸⁹

HvKp's abundant and viscous capsule also influences their interactions with the epithelial cells, mainly by decreasing adhesion and internalisation.^{190–194} Although not demonstrated to date, this could contribute to the immune system avoidance, as *HvKp* is unimpeded and therefore less accessible to immune cells.

Of note, other bacterial factors have also been identified in immune evasion of *HvKp*. For example, outer membrane proteins OmpK36 and OmpA decrease chemotaxis and phagocytosis by both neutrophils and macrophages.^{191,195} Similarly, O-polysaccharide of LPS seems to hinder the TLR signalling pathway in macrophages.¹⁹⁶

1.6 Pathophysiology of HvKp-PLA

Very little is known about the mechanisms used by HvKp to cause community-acquired primary invasive liver abscesses. Each HvKp virulence factor mentioned above has been identified and characterised in various experimental settings, but we lack clear insights about their precise role and importance in the pathophysiology of HvKp-PLA. For instance, if hypercapsule and HMV have been clearly recognised as crucial virulence factors promoting dissemination and virulence in animal models, no straightforward data about their precise function and interactions in the development of HvKp-PLA is available.

A striking point is that most studies support the dissemination of HvKp by translocation to the portal bloodstream following gut colonisation.^{197–199} The colonisation of the gut is not a pathological condition, as HvKp behaves as a coloniser until it eventually translocates through the gut and reaches the liver via the portal circulation.¹⁴⁸ Crucially, the pathogenicity factors compromising the integrity of the gut and facilitating this switch from commensal to pathogen are unknown and poorly explored. Thus far, a few factors have been identified in the different phases of HvKp-PLA formation, as discussed below.

1.6.1 Gut colonisation

Three main factors specific to HvKp have been demonstrated as enabling HvKp to colonise the gut. First, colibactin has been shown as contributing to HvKp virulence through different mechanisms, including increased gut colonisation. This was first

described with *E. coli*^{158,200}, but also with *Kp*, following *in vivo* murine studies of introduction of Hv*Kp* through the orogastric route.¹⁶⁴

Second, as mentioned above, the hypermucoviscous phenotype also seems to enhance Hv*Kp*'s gut persistence, even if its role in initial colonisation has not been defined.^{150,201,202} Finally, the capsule has recently been shown as promoting both gut colonisation and persistence in a murine model.¹⁴⁸

1.6.2 Translocation into the portal bloodstream

This represents the first and most crucial step of Hv*Kp*'s pathogenicity. As described before, the normal gut-vascular barrier function (**Figure 1.13**) is provided by the vascular endothelium, the intestinal epithelium (firmly attached by tight junctions, preventing paracellular leakage), the intestinal microbiota, the outer & inner mucus (produced by goblet cells) and the immune cells (Plasmacytes secreting immunoglobulins (Ig), Paneth cells secreting antimicrobial peptides, mesenteric lymph nodes and M-cells in connection with antigen-presenting cells (dendritic cells) and lymphocytes in Peyer's patches).²⁰³

The mechanism used by Hv*Kp* to translocate is largely unknown. One study available to date, using *Kp* strains "responsible for systemic infection" (without making clear whether they have definite Hv*Kp* features) and Caco-2 cell line (representing intestinal epithelium, see below) *in vitro* has shown that *Kp* passes across the epithelium via a transcellular pathway, without disrupting tight junctions.²⁰⁴

Using a signature-tagged mutagenesis approach with *HvKp* (CG43, K2 strain), Tu et al. have identified 28 transposon mutants for a putative type III biofilm fimbrial protein (*mrkC*) and a two-component system favouring capsule production (*kvgA-kvgS*) which exhibit reduced lethality and dissemination to the liver and spleen in an intraoral infection model.²⁰² Whilst these mutants identify specific factors as necessary for dissemination to the liver and spleen, it is not clear if they are relevant for the formation of liver tropism and abscess formation.

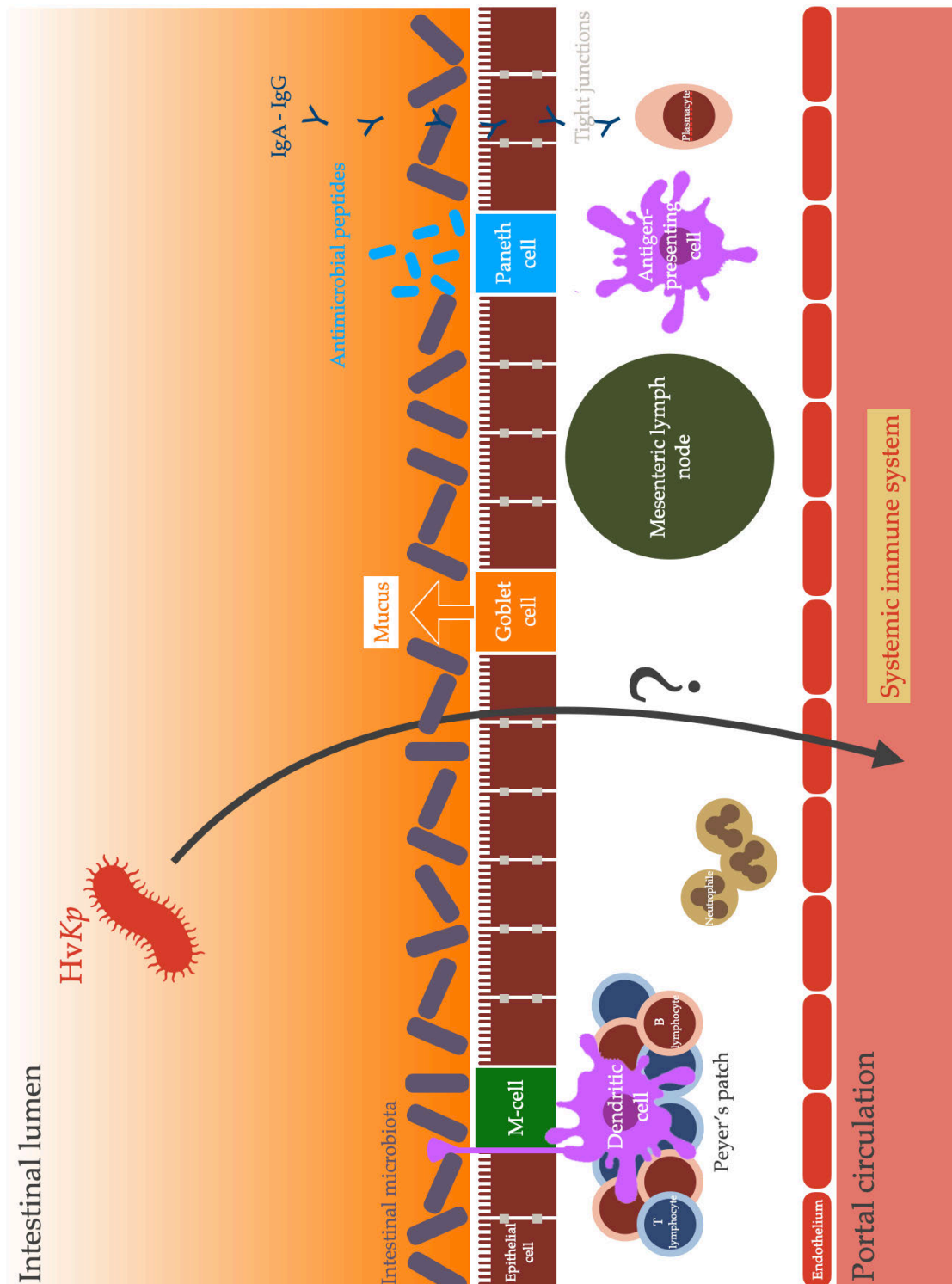


Figure 1.13. Schematic representation of the gut barrier that SGH10 needs to cross to reach the portal circulation.

HvKp faces the mucus (secreted by goblet cells), the microbiota, antimicrobial peptides (secreted by Paneth cells), IgA and IgG antibodies (secreted by plasmacytes), lymphoid tissue and dendritic cells (grouped in Peyer's patches) and the gut epithelial and blood vessel endothelial barriers.

1.6.3 Access to the liver and abscess development

Despite clear liver tropism, there is currently no data which specifically demonstrates host-HvKp interactions using the liver as the model organ. With regards to the liver, one potential scenario is a “Trojan-horse” strategy, where HvKp, surviving phagocytosis, use polynuclear neutrophils as a reservoir and shuttle to the liver. For liver damage *per se*, only colibactin has been proposed as a potential candidate, given its proven genotoxicity.¹⁶⁴ Very interestingly, Kupffer cells, liver resident macrophages, have recently been shown as highly capable of killing HvKp, despite its previously described ability to avoid phagocytosis, making it even more difficult to establish its precise role in pathogenicity.²⁰⁵

Overall, this considerable lack of data describing the pathophysiology of HvKp is a compelling argument for developing a model of infection that includes both the intestinal and hepatic components.

1.7 *In vitro* model of HvKp to mimick infection along the gut-liver axis

In vitro models provide valuable insights into biological processes, allowing controlled, precise and replicable experiments without requiring animal testing. They offer a clear and straightforward understanding of both bacterial and eukaryotic cells and are therefore a perfect tool for preliminary stages of research. Such a model to study HvKp-PLA formation would therefore be desirable.

As previously described, the prominent feature of this process is HvKp's ability to switch from commensal in the gut to pathogen in the liver. To date, no data about interactions between "unambiguous" HvKp (e.g. SGH10) and gut or liver cells is available. A good *in vitro* HvKp infection model should have two major aspects:

- (1) Cells which are representative of the different types HvKp will encounter in the translocation from the gut to the liver (i.e. intestinal, endothelial and liver cells).
- (2) Innovative techniques for assessing bacterial interactions and effect on these cells and their barrier function. In this regard, ECIS (Electrical Cell Impedance System), a cutting-edge impedance-based assay for assessing gut and liver cells culture characteristics and dynamics, is useful to dissect the role of these different factors.

1.7.1 ECIS

The ECIS Z θ TM system by AB Biosciences, which was initially developed by Nobel prize laureates Ivor Giaever and Charles Keese in the 1980s, is a real-time, non-invasive assay allowing precise assessment of eukaryotic cell layer characteristics over time. ^{206,207}

As shown in **Figures 1.14A, B and C**, it utilises 8-well plates containing gold electrodes in which eukaryotic cells are grown and monitored following application of an alternating current (AC) at low voltage (V) yielding a measure of impedance (resistance against the applied electrical current). More specifically, Figure **1.14B** shows that this AC encounters a resistance of 1M Ω , producing a minor 1microAmp current. The main idea is that the impedance measures the resistance faced by this AC, primarily when the cell membrane serves as an insulating barrier. As the cellular layer reaches confluency, the impedance rises due to the current's need to navigate around or between cells.

ECIS mainly stands out amongst other impedance-based assays (e.g. TEER (see below), CNBIO, xCelligence™, CellKey™, and Bionas Discovery™) by its unique capability to separate the data into different biologically meaningful components, i.e. tight junctions state, cell adhesion to the substrate and overall cell membrane integrity. This is achieved by applying different frequencies to measure impedance, revealing the various pathways taken by the current. As illustrated in **Figure 1.14D**, at low frequencies (4kHz), the current will flow beneath the cells and in the spaces between them, reflecting cell adherence to the substrate and the integrity of tight junctions. At higher frequencies (64kHz), the current passes through the cell membrane, reflecting the global cell viability.^{208,209} The deconvolution of impedance measurements into tight junction integrity and membrane capacitance provides an immediate snapshot of host-pathogen-mediated cell disruption.

ECIS measurements have already been used to underscore the onset of damage on both liver and gut cells lines, but also the effect of bacteria on other eukaryotic cell types. For instance, it has been used in the past to demonstrate that chlorpromazine's liver toxicity was due to the damage of liver cells membrane integrity and initiation of a pro-inflammatory response.²¹⁰

With regards to the assessment of bacterial effect using ECIS, a study compared the effect of group A *Streptococcus* isolated from various body sites on human brain microvascular endothelial cells, showing that they exerted the same toxicity on the cell membrane integrity.²¹¹ Interestingly, a study also demonstrated a distinctive effect of LPS depending on the endothelial cell line incubated with, with significantly greater toxic effect on the pulmonary ones.²¹²

Finally, ECIS has been described recently, and for the first time in the literature, as a reliable tool to assess bacterial virulence. Using human alveolar basal epithelial cells (A549) at the single frequency of 4kHz, Nahid et al. (2020) highlighted differences in global cell toxicity between *Pseudomonas aeruginosa*, extended spectrum beta-lactamase producing *Escherichia coli*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant *Enterococcus spp.*²¹³ More specifically, they were able to show that *Pseudomonas aeruginosa* was leading to a lower decrease in that average resistance compared to the three other strains, therefore stated as less virulent.

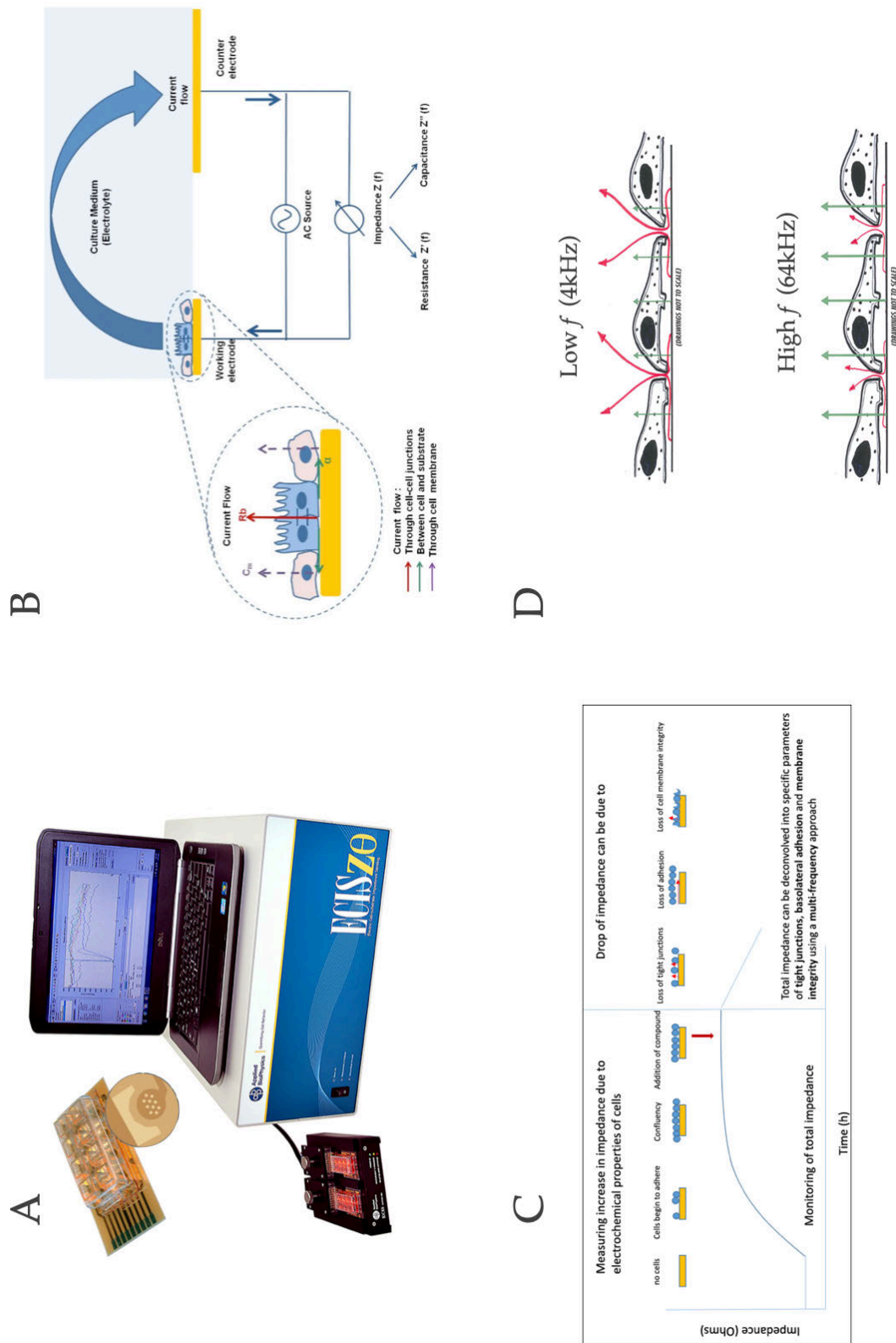


Figure 1.14. A. Picture of the ECIS system. B. Current flow in detail, reproduced from ²¹⁴. C. Technique and approach of ECIS Technology, from ²⁰⁹. D. Difference of current passage depending on the frequency.

1.7.2 Experimental cell lines of the gut-liver axis model

1.7.2.1 *HepaRG*

The HepaRG cell line was isolated from a hepatocellular carcinoma in a 30 year old female, infected with hepatitis C.²¹⁵ They are a grade I differentiated hepatocellular carcinoma cell line containing both hepatocytes and cholangiocyte-like cells, which makes it an outstanding model to mimic the entire hepatic system.²¹⁶ Presenting the same phenotype/function and retaining the characteristics of differentiated liver cells, they are highly comparable to primary human hepatocytes, with the limitation that they don't include immune cells such as Kupffer macrophages.²¹⁷

We recently demonstrated that HepaRG were relevant to study the gut-liver axis function, by performing a study investigating the toxicity on metabolism of paracetamol using both Caco-2 gut cells (see below) and HepaRG in the ECIS environment. We could establish that paracetamol was initially tightening gut cells tight junctions, and that this metabolism was subsequently mitigating its liver cells toxicity.²¹⁸

Likewise, a study showed that gut microbial transformation of the human carcinogen MelQx significantly decreased its toxicity on HepaRG.²¹⁹

1.7.2.2 *Caco-2*

Caco-2 is a human gut epithelial cell line, cultured as a model of barrier function mainly because it exhibits polarity and tight junctions.^{220,221} Crucially, it is capable of passive transcellular and paracellular transport, comparable to *in vivo* rat jejunum and colon.^{220,222} These features make it ideal as an *in vitro* model of the intestinal epithelial

barrier and for assessing altered membrane function. In the context of the gut-liver axis, many models using Caco-2 cells have been described to date. For instance, a team from Japan has recently used them to develop a system mimicking the gut-liver axis, consisting of two chambers connected to each other with a bidirectional circulation channel. This model was further validated by applying LPS on the Caco-2 cells, observing the transmission of inflammatory proteins to the hepatic chamber.²²³ Another example involved the use of both Caco-2 and HepaRG cell lines to display the toxicity on liver cells of vesicles derived from intestinal cells generated by alcohol consumption.²²⁴

1.7.2.3 C3A

These cells, derived from HepG2 cells, are commonly used to model liver cells. The main differences from HepaRG and human hepatic cells is that they don't comprise biliary tract cells and have less metabolism pathways.²¹⁷ Also, compared to primary human hepatocytes, C3A have been shown to have a less clearly defined polarity.²²⁵

1.7.2.4 HUVEC

HUVEC (Human Umbilical Vein Endothelial Cells) are primary endothelial cells derived from the veins of human umbilical cords.²²⁶ Frequently used in vascular research, these cells serve as a model for studying the endothelial function, angiogenesis and atherosclerosis. HUVEC have been shown to respond to cytokines, growth factors, and shear stress, making them a great *in vitro* model for endothelial cells.²²⁷

While an *in vitro* model using ECIS and the eukaryotic cell lines described above would allow many aspects of *HvKp* infection along the gut-liver axis to be dissected and

elucidated, it also has limitations, making an *in vivo* infection model desirable. The 5 most commonly used models for studying host-pathogen interactions in bacterial infections are mice, pigs, *Caenorhabditis elegans*, *Galleria mellonella* larvae and zebrafish larvae.

1.8 The zebrafish model

Zebrafish (ZF) larvae (ZFL) have been used for many years as an *in vivo* model in research, due to their rapid development, their transparency, simple immune system and general organ organisation.^{228,229} The fact that ZFL genes are 80% homologous with humans' and that their genome is easily modifiable also allows their use to study the role of various host features.²³⁰ The ZF has also been extensively used as a bacterial infection model, with the limitation that they need to be maintained at 28°C, not ideal for bacterial growth and stability.^{231,232} As summarised in **Figure 1.15**, various routes of infections have been described to date. Interestingly, ZFL develop a mouth, a gut and a liver from three days after hatching, making them a particularly suitable support to study the gut-liver axis infections *in vivo*. As the key stage in the pathophysiology of HvKp is the colonisation of the intestine followed by translocation to the portal system, the 2 most relevant routes for infecting the ZFL with HvKp are precisely those that allow the bacteria to reach and colonise the intestine, i.e. microgavage and immersion.

Immersion is the easiest route of infection, as it simply involves adding bacteria to the culture medium in which the ZFL incubate. It has the advantage of simplicity, but the disadvantage that (1) the bacteria can also reach other orifices or be deposited on the skin, and (2) the quantity of bacteria ingested and therefore their pathogenic effect seems variable, causing a reproducibility issue.^{233–235}

Microgavage, on the other hand, is much more laborious, but more precise and reproducible.²³⁶ This was illustrated by a study performing this technique to

demonstrate how *Vibrio cholerae* could invade the intestine using its type VI secretion system.²³⁷

Neither of these models has been used to investigate HvKp's pathophysiology to date. Yet, a study has tried to use ZFL to evaluate the virulence of Kp.²³⁸ Three Kp strains, including one HvKp, were used either in an immersion infection or directly injected into ZFL's caudal vein. The immersion route showed no lethality but did demonstrate Kp's gut colonisation differences between strains, although the microbial factors necessary for colonisation were not evident. Infection of ZFL by injection into the tail vein, on the other hand, enabled the difference in virulence between strains to be visualised.²³⁸

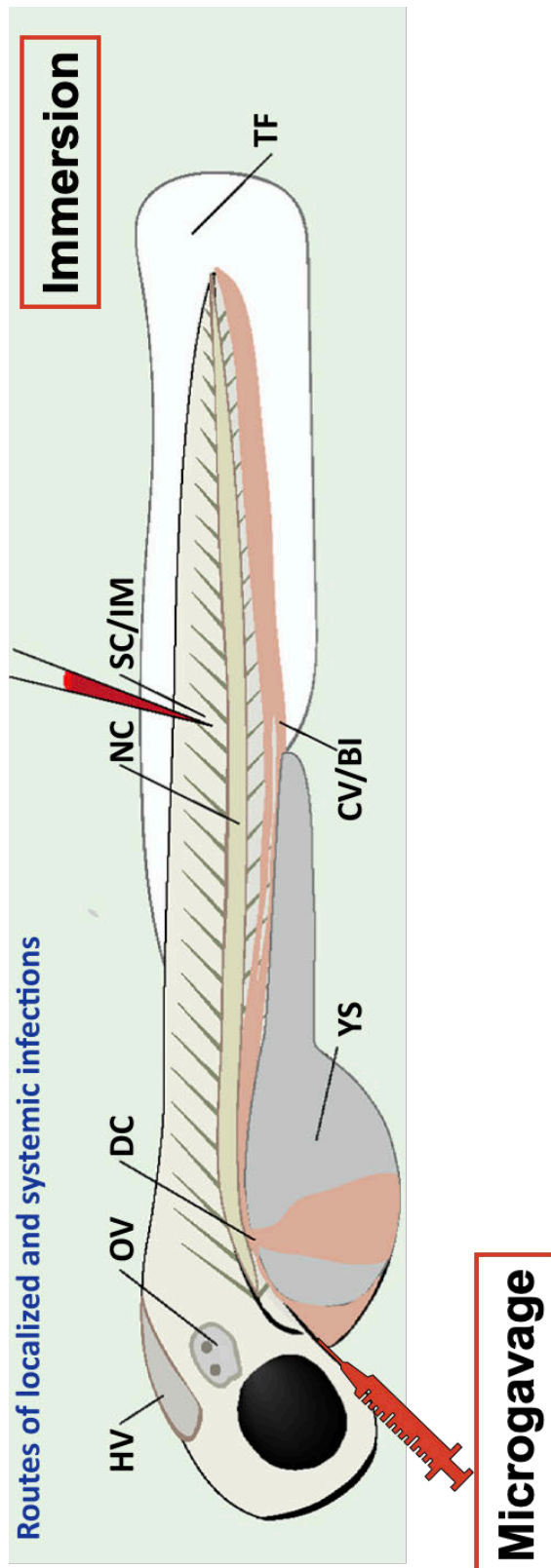


Figure 1.15. Routes of zebrafish infections. Adapted from ²³⁹.

BI, blood island; CV, caudal vein; DC, duct of Cuvier; HV, hindbrain ventricle; IM, intramuscular; NC, notochord; OV, otic vesicle; SC, subcutaneous; TF, tail fin; YS, yolk sac

1.9 Aims/hypothesis

As discussed, the current published literature provides no definitive causes explaining the microbial switch which facilitates the very disparate lifestyles of “gut-located *HvKp*” and “pathogenic liver abscess *HvKp*”. Specifically, the dynamics of host-pathogen interaction at the cellular level are widely unknown. To address these knowledge gaps, this study seeks to dissect the mechanistic details of both host and microbial factors which govern *HvKp* colonisation and infection.

1.9.1 Hypothesis

The hypothesis of this PhD project is that specific host-pathogen interactions along the gut-liver axis disrupt cellular integrity, thereby facilitating transmission from the gut to the liver and transition from commensal to pathogen.

1.9.2 Aims

To address the hypothesis, the aims of our research were to:

1. develop and implement a complex *in vitro* model based on the ECIS technology to explore the interactions between gut/liver cells and *HvKp*;
2. use this model to elucidate the mechanistic, kinetics and interconnections of the specific effects of *HvKp* along the gut-liver axis
3. establish the role of key microbe-associated virulence factors in the pathophysiology of this *HvKp*-PLA

The expected results should allow us to address the host-pathogen dynamics along the gut-liver axis and the factors promoting the transmission and proliferation of these

species *in vivo*. The ultimate aim is to exploit the data generated to identify diagnostic targets and/or immune-therapeutic interventions to limit HvKp-PLA.

Chapter 2: Material & Methods

2.1 Bacterial work

2.1.1 Bacterial Growth Media

2.1.1.1 *LB agar*

LB agar was made by adding bacteriological agar (7.5 g), sodium chloride (5 g), tryptone (5 g) and yeast extract (3.5 g) to 500 ml of distilled water, then autoclaved. Agar plates were fresh before each use. LB Broth-Lennox was made from premade mix obtained from Formedium (Formedium - Reference: LBX0102) and was prepared according to the manufacturer's instructions.

2.1.1.2 *M9 minimal media*

M9 minimal media was made by mixing 20 ml of sterile resuspended 5x M9 salts (Sigma-Aldrich - Reference: M9956) with 2 ml 20% glucose (filter sterilised), 200 µl of 1M MgSO₄, 10µl of CaCl₂, 2 ml of 50X MEM amino acids (Gibco - Reference: 11130051) and 1 ml of 100X MEM non-essential amino acids solution (Gibco - Reference: 11140035), then topped up to 100 ml with sterile water.

2.1.2 Storage of strains

We first streaked bacterial strains onto LB agar plates, incubated at optimal temperatures overnight to isolate individual colonies. A single colony was then picked and transferred into 5 ml of LB broth, incubated shaking overnight. The next day, we centrifuged 1 ml of the broth culture at 12,000 X g to pellet the cells, that were then resuspended in 900 µl of fresh LB medium and 110 µl of sterile 90% glycerol. The prepared samples were then stored at -80°C.

2.1.3 Bacterial strains used

Name	Organism/ST	Description	Antibiotic susceptibility	Reference
SGH10	<i>K. pneumoniae/ST23</i>	HvKp reference strain, obtained from Gan team, Singapore.	Amp R, Cef S, AMC S Cm S, Km S, Tig S, NA S, Col S	Lam et al. (2018) ¹⁰⁷
SGH10-p	<i>K. pneumoniae/ST23</i>	SGH10 without the virulence plasmid (thus lacking <i>iuc</i> (aerobactin), <i>iro</i> (salmochelin), <i>rmpA</i> (regulator of mucoidy phenotype A), <i>rmpA2</i> , <i>rmpC/D</i> genes), previously generated in our lab	Amp R, Cef S, AMC S Cm S, Km S, Tig S, NA S, Col S	Schneiders' lab
Ecl8	<i>K. pneumoniae/ST375</i>	Multi-Drug Resistant Kp strain, yet K2 serotype and containing a plasmid coding for aerobactin and salmochelin.	Amp R, Cef S, AMC S Cm S, Km S, Tig S, NA S, Col S	George, Hall and Stokes, (1995) ²⁴⁰

ATCC 43816 - KPPR1	<i>K. pneumoniae/ST23</i>	K2 strain, used to study hypermucoviscosity and hypercapsulation	Amp R, Cef S, AMC S Cm S, Km S, Tig S, NA S, Col S	Lawlor et al. (2005) ²⁴¹
Ecl8 + GFP	<i>K. pneumoniae/ST375</i>	Ecl8 with Green-Fluorescent Protein (GFP) plasmid (pRSMGFP), with Chloramphenicol (Cm) selection cassette	Amp R, Cef S, AMC S Cm R, Km S, Tig S, NA S, Col S	This study
SGH10 + GFP	<i>K. pneumoniae/ST23</i>	SGH10 with Green-Fluorescent Protein (GFP) plasmid (pRSMGFP), with Chloramphenicol (Cm) selection cassette	Amp R, Cef S, AMC S Cm R, Km S, Tig S, NA S, Col S	This study
SGH10-p + GFP	<i>K. pneumoniae/ST23</i>	SGH10-p with Green-Fluorescent Protein (GFP) plasmid (pRSMGFP), with Chloramphenicol (Cm) selection cassette	Amp R, Cef S, AMC S Cm R, Km S, Tig S, NA S, Col S	This study

Table 2.1. Name and description of different bacterial strains

S = Sensitive. R = Resistant.

Amp = Ampicillin. Cef = Cefuroxime. AMC = Amoxycillin – Clavulanate. Cm = Chloramphenicol. Km = Kanamycin. Tig = Tigecycline. NA = nalidixic acid. Col = Colistin.

2.1.4 Primers & Plasmids

Plasmid	Description	Genotype or characteristics	Reference
pTOF25	Used for the generation of the replacement cassette	Cm ^r Km ^r , 30°C	Merlin, McAteer and Masters, (2002)
pTOF3	Contains <i>aph</i> for insertion between NoNi and CoCi	Km ^r , 30°C	Merlin, McAteer and Masters, (2002)
pRSMGFP+	Plasmid carrying a Green Fluorescent Protein	37°C	/
pTOF <i>rmpD</i> NoCo<km>	Plasmid for the deletion of <i>rmpD</i>	Cm ^r , Km ^r , 30°C	This Study
pTOF <i>rmpA</i> NoCo<km>	Plasmid for the deletion of <i>rmpA</i>	Cm ^r , Km ^r , 30°C	This Study
pTOF <i>wcaJ</i> NoCo<km>	Plasmid for the deletion of <i>wcaJ</i>	Cm ^r , Km ^r , 30°C	This Study

Table 2.2. Name and description of plasmids used for this work

Table of primers used for this work can be found in **Appendix S1**.

2.1.5 Antibiotics

Antibiotic	Solvent	Concentration
Kanamycin	Distilled Water	50mg/ml
Chloramphenicol	70% Ethanol	50mg/ml
Ampicillin	Distilled Water	100mg/ml

Table 2.3. Name and description of antibiotics

2.1.6 Plasmid removal protocol

The plasmid-removed SGH10 (SGH10-p) was generated in our lab by the former PhD student Thomas Stanton, as described by Gonzalez-Iglesias et al.²⁴² Briefly, SGH10 was subjected to an electric pulse of 12.5 kV/cm, 1000 V and 25 mF (GenePulser Xcell™, BIO-RAD®). We then performed 6 cycles of plating and single-colony subculturing in LB broth at 37°C. The absence of the plasmid was confirmed by *rmpA2* PCR and selection on potassium tellurite plates (10ml/l of 3.5% solution).

2.1.7 Growth curves of bacteria

2.1.7.1 *Relation between optical density (OD₆₀₀) and bacterial growth (cfu/ml)*

Bacteria were grown overnight in LB broth at 37°C, with shaking. They were then diluted (1:100) in fresh LB broth and kept in the shaking 37°C incubator. Every 30 minutes for 3.5 hours, OD₆₀₀ was measured using BIO-RAD® SmartSpec™ 3000 and the broth was plated (with appropriate dilution on LB agar plates). The plates were stored in the 37°C incubator overnight, and the colonies were counted the next day.

2.1.7.2 Growth in tissue culture media/PBS

The growth of our strains in tissue culture media and PBS was assessed by using CLARIOstar™ plate reader (BMG Labtech), measuring OD₆₀₀ as a function of time. Bacteria were grown overnight in LB broth at 37°C, with shaking. They were then diluted in fresh LB broth to obtain an OD of 0.1, spun down at 10,000 *X g* for 10 minutes, and resuspended in tissue culture media (MEME + for Caco-2 or MMM (without Streptomycin) for HepaRG, see details in **Table 2.6** below) and PBS. This suspension was then transferred in a 96-wells plate (in triplicates) and the OD₆₀₀ was recorded every 30 minutes for 5 hours. The suspension was plated at T0 and T5 with appropriate dilutions to confirm growth and viability.

2.1.8 Quantification of capsule production

These protocols were adapted from Favre-Bonte et al.²⁴³ and Dorman et al.²⁴⁴

2.1.8.1 *Percoll stage*

Bacteria were grown in overnight cultures at 37°C in LB broth. The culture was then centrifuged at 4500 rpm for 5-10 min, and the cells were resuspended in 1 ml of PBS. A Percoll™ (GE Healthcare - Reference: 17-0891-02) gradient was prepared in a 50 ml Falcon tube, using 10%, 30% and 50% fractions (Percoll™:H₂O; % v/v), of 2 ml each (see **Figure 2.1**). 600 µl of the resuspended cells were applied on the top of the column, which was subsequently centrifuged for 30 minutes at 4500 rpm at 4°C. The location of the pellet was then assessed by eye.

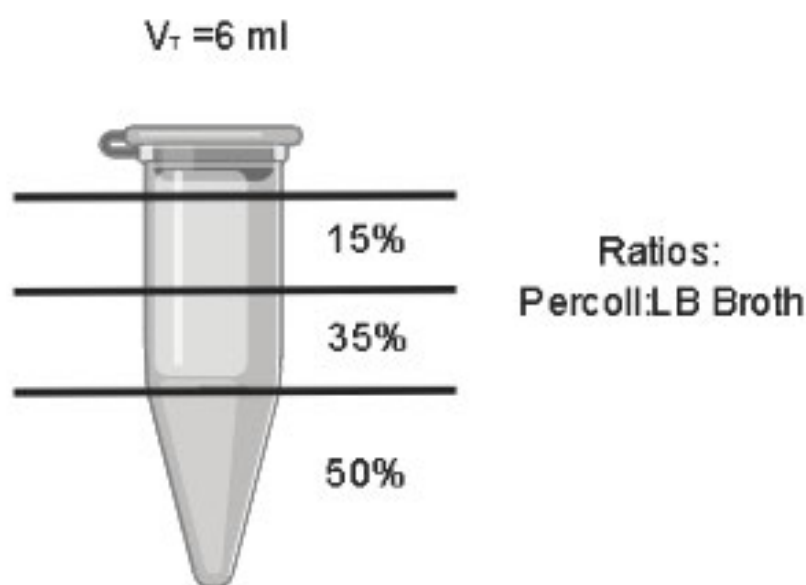


Figure 2.1. Illustration of the Percoll gradient.

2.1.8.2 *Uronic acid assay quantification*

Either 600 µl of the bacterial layer from the Percoll gradient or 2 ml of overnight culture was spun down and washed three times with PBS, to be finally resuspended in a final

volume of 1.5 ml of PBS. After measuring its OD₆₀₀ for further normalisation of the results, 500 µl of this mixture was transferred into a fresh Eppendorf tube, where 100 µl of capsule extraction buffer (see composition below) was added. This was then incubated at 50°C for 20 minutes, and centrifuged at 13,000 rpm for 5 minutes at 4°C. 300 µl of the supernatant was collected into a clean Eppendorf tube and washed with 1.2 ml of absolute ethanol for 30 minutes at 4°C. Following centrifugation at 13,000 rpm for 20 minutes at 4°C, the supernatant was removed and the pellet (transparent and difficult to distinguish) was air dried for 10 minutes. It was then resuspended in 100 µl of distilled water, and 600 µl of capsule quantification buffer (see composition below) was added. This mixture was incubated on ice for 10 minutes then at 95°C for 5 minutes, then on ice again for a further 15 minutes. After these incubations, 20 µl of 0.15% 3-hydroxybiphenyl in 0.5% sodium hydroxide was mixed into each sample, and the absorbance was measured at 520 nm. The glucuronic acid concentration was extrapolated using GraphPad Prism™ 9.0 from a standard curve made according to the same technique but using samples with a known glucuronic acid concentration. The Capsule Extraction Buffer consisted of 500 mM citric acid (Sigma-Aldrich - Reference: 251275) and 1% (w/v) Zwittergent 3-10 (Merck – Reference: 693021) in pH 2.0.

The Capsule Quantification Buffer consisted of 0.0125 M sodium tetraborate (Na₂[B₄O₅(OH)₄]·8H₂O, Sigma-Aldrich – Reference: 221732) in concentrated sulfuric acid (H₂SO₄).

2.1.9 Quantification of mucoviscosity levels

The mucoviscosity of our strains was performed using low-speed centrifugation of our cultures, as described by Walker et al.²⁴⁵ Briefly, we grew our strains in M9 medium

overnight. Cultures were then diluted to reach 1OD₆₀₀/ml and centrifuged at 1,000 X g for 5 min. We then aspirated the supernatant and transferred it to a cuvette to measure its OD₆₀₀.

2.1.10 Chrome Azurol S (CAS) experiments to determine siderophores production

In this assay, plates contain iron which is part of complex (Fe-CAS-hexadecyltrimethylammonium bromide) that appears blue. When the iron is captured by siderophores, the plate color switches from blue to orange. CAS agar plates were made by dissolving 6 g of NaOH and 30.24 g of 1,4-piperazinediethanesulfonic acid (PIPES) (free acid) (Sigma-Aldrich) into 750 µl of water. 100 ml of sterilised 10× M9 salts and 15g (Double strength) of Bacto agar (Formedium) were added to the mix. We then added 30ml of 10% casamino acids (prepared in point 1), 10 ml of filter-sterilised 20% glucose, 1 ml of 1M MgCl₂, 1 ml of 100 mM CaCl₂, 1 ml of 1% nicotinic acid and 1 ml of 1M MgSO₄·7H₂O. Finally, 100ml of Fe-CAS-HDTMA solution was added to the mix. This was then poured into a petri dish and solidified overnight at room temperature. 5 µl of overnight culture of bacteria normalised to an OD₆₀₀ of 1.5 was then added to the plate and incubated at 30°C for 18 hours. The diameter of the colour change area was measured using ImageJ.

2.1.11 RNA extraction and Quantitative real time PCR (qPCR)

Bacteria were grown overnight in LB broth, at 37°C, shaking. Overnight cultures were then sub-cultured in fresh LB (dilution 1:100) and grown until reaching an OD₆₀₀ of 0.6-0.8. The RNA was extracted using Monarch Total RNA Miniprep Kit (New England Biolabs – Reference: T2010S), according to manufacturer's instructions. qPCR

reactions were set up using the GoTaq® 1-Step RT-qPCR System Sybr Green (Promega – Reference: A6020), using standardised amount of RNA (25 ng in 10 µl reaction volume). The reactions were placed in 96 well qPCR reaction plates (Starlab – Reference I1402) and plates were run in a Roche LightCycler 96® machine. Ct (Cycle threshold) values were extracted, $\Delta\Delta C_t$ and fold changes were calculated and analysed using Microsoft Excel®.

2.1.12 Genetic manipulation of our strains

We used the allelic exchange method described by Merlin, McAteer and Masters²⁴⁶ to generate mutants of SGH10 (Figure 2.2, showing the example of *rmpD*). Primers were designed to amplify ~1 kbp regions flanking the gene of interest, with the addition of specific restriction sites SmaI/SalI-NotI using Q5 High Fidelity Polymerase (NEB, details below. Primers sequence in **Table S1, appendix**). Following the amplification of individual flanking regions, an overlap PCR was set up to generate a ~2 kbp NoCo fragment lacking the gene of interest. This DNA fragment was purified, ligated to the pJET 1.2/blunt linear cloning vector and transformed into *E. coli* DH10 β . Colony PCR was undertaken to ascertain the correct insertion of the DNA fragment. Following restriction digest of the pJETNoCo recombinant and the pTOF24/25 vector, ligation and transformation into *E. coli* DH10 β was performed.

Both pTOF24/25NoCo and pTOF3 (containing the Km^r cassette) underwent digestion with NotI FD enzyme and were then subjected to electrophoresis on a 1% agarose gel. We isolated and purified the 1252 bp band of the Km^r cassette from pTOF3, along with the band of the restricted pTOF24/25NoCo. Following ligation and transformation, as

done previously, we confirmed the presence of the pTOF24/25NoCo-Km^r, extracted it, and stored it at -20°C. This construct was subsequently introduced into SGH10, which were made electrocompetent as described above, using an Eporator™ (Eppendorf). Post-electroporation, the cells were incubated overnight on LB agar supplemented with kanamycin at 30°C. From this, six robust colonies were selected and cultured in LB broth with chloramphenicol, also at 30°C and 200 rpm. Further steps involved diluting these cultures by a 10⁴ and 10⁶ factor, spreading them on LB plates containing chloramphenicol, and incubating overnight at 30°C and 42°C. We transferred some colonies onto LB agar containing either kanamycin or chloramphenicol and incubated them overnight at 30°C. PCR screening of Km^rCm^s colonies was conducted to check for the Km^r cassette, aiming to confirm allelic exchange. Colonies that tested positive were cultured in LB with kanamycin at 37°C and 200 rpm, and then preserved in glycerol at -80°C.

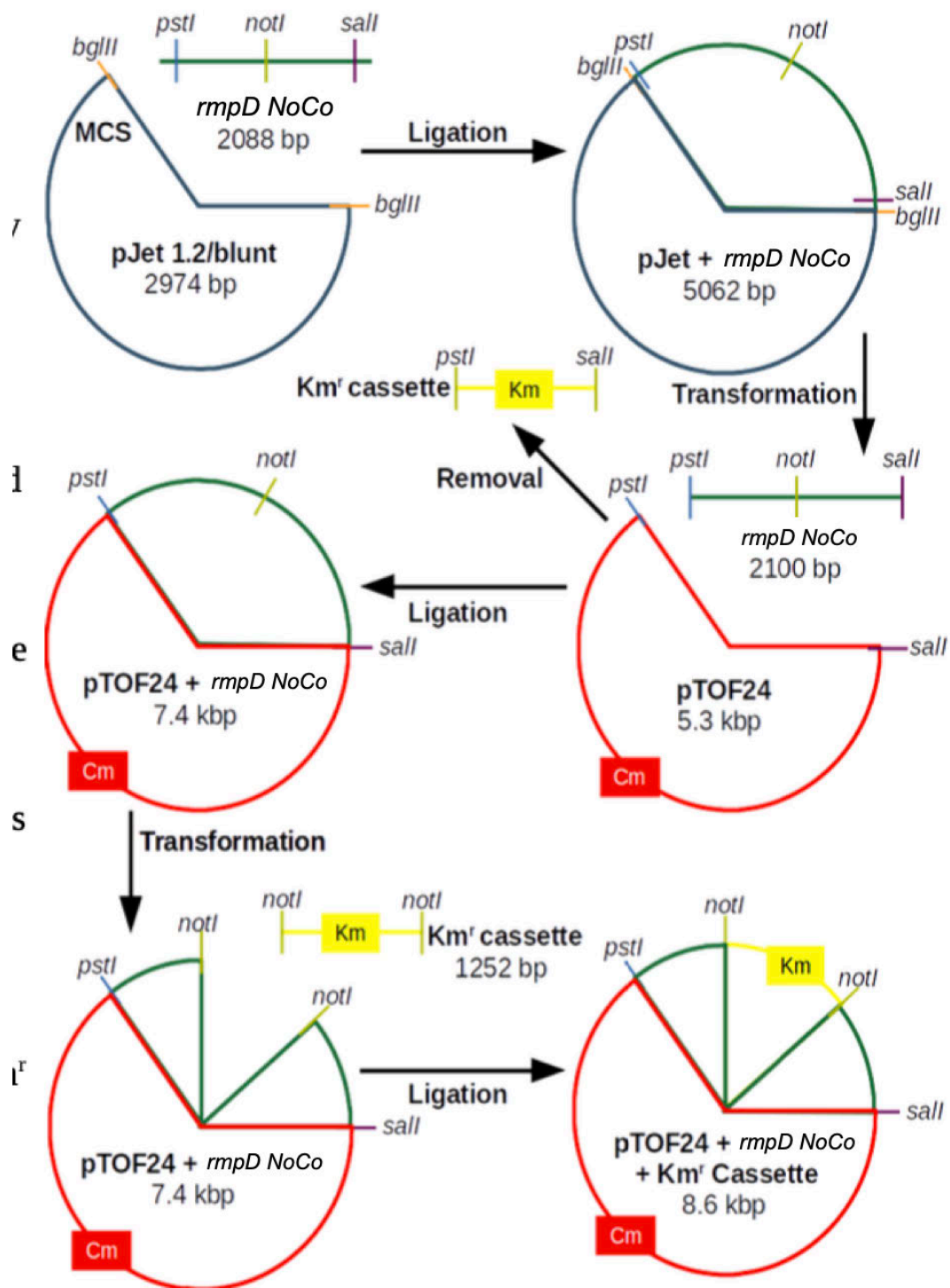


Figure 2.2. Construction of the pTOF plasmid for further deletion of *rmpD*.
Credits to Thomas Stanton (unpublished).

2.1.12.1 Q5 High Fidelity PCR

Amplification of all the fragments needed for the allelic exchange was carried out using the Q5 High Fidelity PCR kit (New England Biolabs).

Component	Volume for 25µl Reaction
5X Q5 Reaction Buffer	5 µl
10 mM dNTPs	2 µl
10 µM Forward Primer	1.25 µl
10 µM Reverse Primer	1.25 µl
Template DNA	2 µl
5X Q5 High Fidelity GC Enhancer	0.5 µl
Q5 High Fidelity DNA Polymerase	0.25 µl
Nuclease Free Water	To 25 µl

Table 2.4 Mix for the Q5 polymerase PCR reaction

Step	Temperature (°C)	Time (seconds)
Initial denaturation	98	30
30 cycles	98	10
	50-72	30
	72	30/kb
Final extension	72	120
Hold	4	∞

Table 2.5 Cycling parameters used for Q5 polymerase

2.1.12.2 *OneTaq colony PCR*

Colony PCR was performed to double-check for the correct position of DNA fragments in the bacterial strains. A colony was picked from an agar plate, transferred into a PCR tube, where the following mix was added: 6.25 μ l OneTaq™ MasterMix 2x, 0.25 μ l forward primer, 0.25 μ l reverse primer and 5.75 μ l Nuclease Free Water. The following cycle was then performed:

Step	Temperature(°C)	Time (seconds)
Initial denaturation	98	30
30 cycles	98	10
	45-68	45
	68	60/kb
Final extension	68	300
Hold	4	∞

Table 2.6 Cycling parameters used for OneTaq

2.1.12.3 *Agarose Gel Electrophoresis*

Amplification or enzyme restriction DNA products were run in 1% w/v agarose gel made in 1 X TBE buffer (1 in 10 dilution of 10 X TBE stock: 90 mM Tris base, 90 mM Boric Acid, 20 mM EDTA), with 1 X Gel Red stain. A 1kb DNA ladder was used. Electrophoresis was carried out in a Bio-Rad Mini sub-cell in 1 X TBE buffer at room temp, 120V for 30. Images were obtained with a Bio-Rad Gel Doc 1000.

2.1.12.4 *Purification of PCR/Restriction Digested DNA*

After electrophoresis, DNA bands of expected molecular weight were extracted and purified using one of the following kits: GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Loughborough, UK), QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and GenElute Gel Extraction Kit (Sigma-Aldrich, Gillingham, UK).

2.1.12.5 *Preparation of calcium competent DH10 β cells*

E. coli DH10 β was grown overnight and sub-cultured (1:100 dilution) into 50 ml of fresh LB broth. This was then incubated at 37°C with shaking at 200 rpm until the culture reached an OD₆₀₀ of 0.6-0.8. This culture was transferred into 50 ml falcon tubes, centrifuged at 4000 $\times$ g at 4°C for 15 min, then washed in 25 ml of sterile ice cold 0.1 M CaCl₂. This was repeated three times. After three washes, the pellet was resuspended in 900 μ l of sterile ice cold CaCl₂ and 110 μ l sterile 90% glycerol. Cells were aliquoted into a 100 μ l volume and stored at -80°C. Contamination was ascertained by streaking an aliquot onto three LB agar containing ampicillin, kanamycin and chloramphenicol and incubated overnight at 37°C.

2.1.12.6 *Preparation of HvKp electrocompetent cells*

The protocol was specially adapted for hypermucoviscous strains, adapted from Walker et al.²⁴⁵ SGH10 was grown overnight at 26°C to minimise capsule production, then sub-cultured (1:100 dilution) into 50 ml of fresh LB broth with 0.7mM EDTA. This was then incubated at 37°C with shaking at 200 rpm until the culture reached an OD₆₀₀ of 0.6-0.8. This culture was transferred into 50 ml falcon tubes, centrifuged at 4000 $\times$ g at 4°C for 15 min, then washed three times in 25 ml of sterile ice cold 10%. The pellet

was resuspended in 1 ml sterile 10% glycerol. 40 µl aliquots were stored at -80°C. Contamination was prevented by streaking an aliquot onto three LB agar containing kanamycin and chloramphenicol and incubated overnight at 37°C.

2.1.12.7 Plasmid DNA Extraction

Plasmid DNA was extracted following the manufacturer's instructions using either GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific, Loughborough, UK), QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), or GenElute 5 Minute Plasmid DNA Miniprep kit (Sigma-Aldrich, Gillingham, UK). All extracted DNA samples were stored at -20°C.

2.1.12.8 Plasmid transformation

a. Heat shock for DH10β

Aliquots of 100 µl of chemical competent cells were thawed on ice, then 50-250 ng of plasmid was added, and the mix was incubated on ice for 30 minutes. This was then heatshocked in a water bath at 42°C for 45 seconds, then placed back into ice for a further minute, before the addition of 1 ml of LB broth and incubation at 37°C shaking for an hour. Cells were then spun at 12,000xg for 3min, resuspended in 100 µl of fresh LB broth and plated in two plates with appropriate antibiotic selection in a 90:10 split and spread.

b. Electroporation

A 40 µl of electrocompetent SGH10 were mixed with 50-100ng of plasmid (volume no more than 5 µl) and transferred to an electroporation cuvette (1 mm gap, Flowgen, SLS), prior to undergoing a 1.8 kV, 200 ohms, 25 µF shock, followed immediately by the addition of 1 ml of SOC (Super Optimal broth with Catabolite repression, Sigma-

Aldrich, Gillingham, UK 15544034) and incubated for 1 hour with shaking at the appropriate temperature. SGH10 were then spun at 12,000xg for 10 min, resuspended in 100 µl of fresh LB broth and plated in two plates with appropriate antibiotic selection in a 90:10 split and spread.

2.2 Cell culture

Except from the Caco-2 and HepaRG cells summarised in **Table 2.7**, all cell lines used in our study, along with their conditions of culture, are found in **Table S2 (appendix)**.

Intermittent microscopy was used to check the viability and morphology of the cells.

Cell	Description	Medium	Seeding density (/cm ²)	Culturing
HepaRG	Human hepatocytes and cholangio-cytes	General Purposed Supplement (GPS): Adding HepaRG [®] Thawing/Plating/General supplement (Biopredic – Reference: [ADD671C]) to 500 ml of Williams E media (Sigma-Aldrich – Reference: A1217601) and 5 ml of GlutaMAX [™] -I (Sigma-Aldrich – Reference: 35050061)	220,000, in GPS	HepaRG were initially plated in GPS, changed to MMM on day 2, continued until use of the cells (day 7-8), changed every 2 days.

		Maintenance & Metabolism Medium (MMM, Biopredic – Reference: [HPRG621]) added to 500 ml of Williams E media (Sigma-Aldrich - Reference: A1217601)		
Caco-2	Human ileocolic cells	Modified Essential Medium, Eagle (MEME+) [Sigma-Aldrich – Reference: M0268]), containing 20% FCS (Fetal Calf Serum) but no antibiotic.	5000, in MEME+	Media was changed on day 1, then every 2 to 3 days. Cells were grown for 10 to 14 days, to ensure tight junctions were formed. Only cells below passage 20 were used.

Table 2.7 Parameters of cell lines seeding and culture for HepaRG and Caco-2

2.3 Infection assays

2.3.1 Determination of the multiplicity of infection (MOI)

We used a MOI of 50 CFU/eukaryotic cell, which was determined as follows: an overnight culture in LB broth was diluted (1:100) in fresh LB, grown to reach an optical density of around 0.8. The concentration of bacteria corresponding to this OD₆₀₀ was then extrapolated, based on growth curves determined previously. This concentration was also checked by plating dilutions of the solution containing the bacteria, counted after incubation at 37°C overnight.

The number of eukaryotic cells in a well was determined by two techniques:

- Flow cytometry analysis: cells were grown in a 96-well plate until day 7 for HepaRG, 10 and Caco-2. They were lifted from the bottom of the plate with 0.05% Trypsin for 20 minutes, then diluted in 200 µl of PBS (Phosphate Buffer Saline, 1 in 10 dilution of 10 x PBS; 25.6 g Na₂HPO₄·x7H₂O, 80 g NaCl, 2 g KCl, 2 g KH₂PO₄). Samples were analysed using a cytometer. 100 µl of sample were acquired, distinguishing accurately the viability of the cells, as well as the presence of aggregated cells.
- Automatic visual count of stained nuclei on EVOS™ microscope (ThermoFisher): cells were grown in a 96-well plate until day 7 for HepaRG, and day 10 for Caco-2. Supernatant was discarded and the wells were washed with PBS. The cells were then fixed with 4% formaldehyde for 30 minutes at 4°C and stained with DAPI (4',6-diamidino-2-phenylindole, binding to DNA, ThermoFisher, D1306) for 30 minutes at 4°C, in the dark. After three washes with PBS, the plates were assessed using the EVOS, to automatically count the nuclei (blue dots).

We then extrapolated the number counted in a limited area to the surface area of the well.

2.3.2 Adhesion assay

Cells were grown on 96-well & ECIS plates until day 7 for HepaRG and day 10 for Caco-2 (time to give a confluent monolayer of cells, with effective tight junctions). Bacteria were grown overnight in LB broth at 37°C, with shaking. They were then diluted (1:100) in fresh LB broth and grown for approximately 90 minutes, until they reached an OD₆₀₀ of around 0.8. They were resuspended in PBS and added to the cells at a multiplicity of infection (MOI) of 50 (see above). After addition of bacteria, HepaRG were centrifuged for 3 minutes at 200 X g and then incubated with bacteria for 30 minutes. Caco-2 cells didn't undergo centrifugation and were statically incubated with bacteria for 90. After incubation, the wells were washed four times with PBS, bacteria were released from the cells by addition of 0.1% Triton X-100 (Sigma-Aldrich – Reference: X100), 5 minutes for HepaRG and 7 minutes for Caco-2, and plated with appropriate dilutions. The plates were incubated at 37°C overnight and the number of colonies was counted the next day. Adherent bacteria were expressed as a percentage of the inoculum.

2.3.3 Invasion assay

The same steps as for the adhesion assay were followed, except that after the first incubation, the wells were washed and a prewarmed medium containing 100 µg/ml gentamicin was added to each well for 2 more hours to kill extracellular bacteria. Internalised bacteria were released with 0.1% Triton X-100 coupled with shaking for 10 minutes and plated as described above.

2.3.4 Microscopy (EVOS) assay

2.3.4.1 *Kp-eukaryotic cells interactions visualisation*

Host-*Klebsiella* interactions were visualised by high-definition microscope (EVOS™, ThermoFisher), an imaging technology discerning chemical or antibody-based staining by fluorescence. Cells were grown on a 96-well plate and infection was performed as described for the adhesion assay. However, the pRSMGFP plasmid was electroporated in the strains to make fluorescent. After infection, the wells were washed four times with PBS, the cells were fixed with 4% formaldehyde for 30 minutes at 4°C and stained with DAPI (nucleus, ThermoFisher – Reference: D1306) and Texas Red™-X Phalloidin (cytoskeleton, ThermoFisher – Reference: T7471) for 30 minutes at 4°C in the dark. All wells were intensively washed, and the plates were observed on an automated microscope EVOS® using different channels (DAPI, GFP, RFP (Red-Fluorescent Protein) and Phase).

2.3.4.2 *Assessment of tight junctions*

To visualise and assess the integrity of tight junctions using the high-definition microscope (EVOS™, ThermoFisher), cells were grown on a 96-well plate and infection with GFP-tagged HvKp was performed as described for the adhesion assay. After infection, the wells were washed four times with PBS, the cells were fixed with 4% formaldehyde for 30 minutes at 4°C, blocked for 60 minutes in blocking buffer (5% fetal calf serum (FCS) and 0.1% Triton™ X-100 in PBS), then probed with ZO-1 primary antibody (ThermoFisher – Reference: 61-7300; 1:1000 dilution in antibody dilution buffer (1% bovine serum albumin and 0.1% Triton X-100 in PBS)) at 4°C overnight. The next day, the wells were thoroughly washed with PBS and

the secondary antibody (Goat anti-Rabbit IgG coupled with Alexa Fluor™ 555 (ThermoFisher – Reference: A-21428; diluted 1:3000 in antibody dilution buffer) was incubated for an hour at 37°C. All wells were intensively washed, and the plates were observed on an automated microscope EVOS® using different channels (GFP, RFP and Phase). Images were analysed using Volocity 7™ software.

2.3.5 ECIS

2.3.5.1 *Simple infection model*

Cells were grown on 8-well ECIS plates for various durations, depending on cell type (see **Table 2.7** and **Appendix S2**). The plates were connected to the ECIS machine (Applied Biophysics Biosciences ECIS Z0™), up to 24 hours prior infection to confirm “plateau” phase, indicating that cells were covering the entire surface of the electrode. Bacteria were grown overnight in LB broth at 37°C, with shaking. They were then diluted (1:100) in fresh LB broth and grown for 90 minutes, to reach an OD₆₀₀ around 0.8. They were spun down and resuspended in tissue culture media and added to the wells at a MOI of 50 (see above).

Impedance, resistance and capacitance at multiple frequencies (ranging from 62.5Hz to 64kHz) were recorded for 24 to 48 hours. The results were exported from the ECIS software into an Excel spreadsheet.

ECIS allows dissection of the cell-pathogen interactions by measuring the variations in cellular electrical resistance in the presence of the pathogen over time. However, if a trend is repeatedly observed, no precise method to quantify it exists. After applying

multiple different methods of analysis, the most reliable and pertinent factor we developed was the “T₅₀ decrease factor” (T₅₀DF), i.e. the time necessary for a 50% reduction of the amplitude of the curve. To calculate the T₅₀DF, we imported our dataset into Rstudio (version 2023.03.0+386), where a script was written for further data management. As demonstrated in **Figure 2.3**, we first normalised the time and resistance values: T0 was set one hour before addition of bacteria and the value of resistance of the plateau phase was defined as “1”. We then calculated the value of the resistance corresponding to half of the amplitude, which was found by dividing the difference between the highest and the lowest value of the normalised resistance. We then extrapolated the time when this value of resistance was recorded.

```

1 library(writexl)
2 library(tidyverse)
3
4 # Load my results Excel file
5 data <- read_xlsx("results.xlsx")
6
7 # Define values for X (= Time when bacteria added) and Y (= Value of resistance of the plateau phase)
8 X <- # Replace with value of time when bacteria added
9 Y <- # Replace with value of resistance just before addition of bacteria
10
11 # Normalise the time and add a column called "normalised_time"
12 data$normalised_time <- data$time - X
13
14 # Normalise the resistance and add a column called "normalised_resistance"
15 data$normalised_resistance <- data$resistance / Y
16
17 # Calculation of the value of resistance that's half of the amplitude
18 T50DF_resistance <- (max(data$normalised_resistance) - min(data$normalised_resistance)) / 2
19
20 # Find the index of the "normalised_resistance" value closest to T50DF
21 closest_index <- which.min(abs(data$normalised_resistance - T50DF_resistance))
22
23 # Get the corresponding "normalised_time" value = T50DF
24 T50DF <- data$normalised_time[closest_index]
25
26 # Get the T50DF result
27 print(T50DF)

```

Figure 2.3. Generic code for calculation of T₅₀DF in Rstudio

The advantage of this method is that it considers both the time at which the effect starts, but also how intense it is (i.e. the steepness of the curve decrease). To calculate

this factor from our data, we first normalised it (divided all the timepoints values by the initial value of the resistance/impedance, so that all curves start from 1), and then plotted the resulting curve of each replicate. The T_{50DF} was always counterchecked geometrically on each individual curve. To note, a condition for the calculation of this T_{50DF} was to have a variation in amplitude of at least 10%, to avoid considering irrelevant variations in resistance values. These T_{50DF} values were then plotted and compared between strains and technical replicates using *t-test* in GraphPad Prism.

2.3.5.2 Infection with heat-inactivated or filtered bacteria

The setup of the ECIS plate was similar as the one described in 2.3.5.1, except that the bacteria, resuspended in cell culture media, were either heat-inactivated (by incubation at 70°C for 30 minutes) or sterile-filtered using a 0.22 mm pore-size filter, before addition to the ECIS wells. Measurements were then recorded for 24 hours. The T_{50DF} was determined as previously described.

2.3.5.3 Interrupted infection

The setup of the ECIS plate and the infection with bacteria were similar to that described in paragraph 2.3.5.1. However, these bacteria were withdrawn from the well after either 2 or 4 hours of incubation. To do so, the medium containing the bacteria was aspirated from the well, filtered through a 0.22 mm pore-size filter, supplemented with kanamycin (50mg/ml) and reinjected into the well, where the electrical parameters were recorded for 24 hours. The T_{50DF} was determined as previously described.

2.3.5.4 Transfer of supernatant from infected Caco-2 cells

As illustrated in **Figure 2.4**, Caco-2 cells were first infected with our strains resuspended in MEME+, at a MOI of 50, in a standard 24-well plate, for 4 hours. The supernatant was then collected, filtered using a 0.22 mm filter, supplemented with kanamycin and transferred to confluent HepaRG grown in wells of an ECIS plate. Measurements were then recorded for 24 hours. The T_{50DF} was determined as previously described.

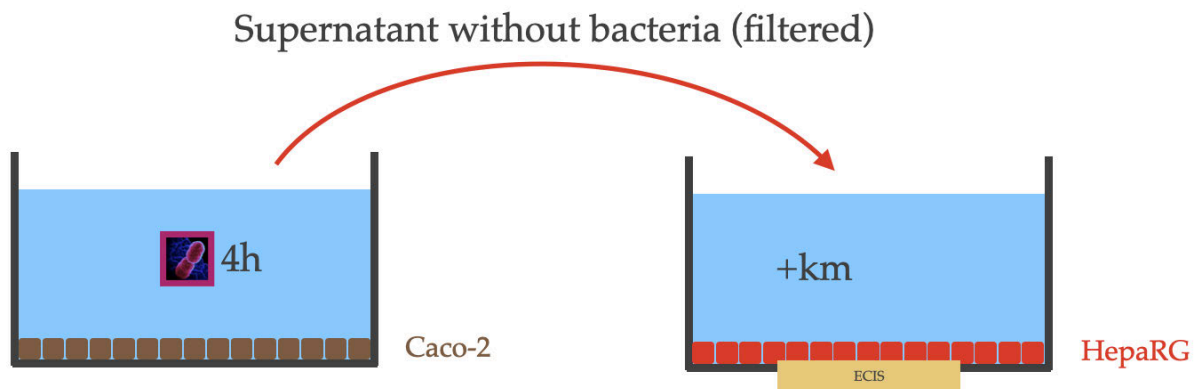


Figure 2.4. Schematic representation of the incubation of HepaRG with the bacteria-free supernatant post-infection of Caco-2 cells, monitored on ECIS

2.3.5.5 Transwell model

Caco-2 cells were seeded in 3 μ m-pore Transwell inserts, placed in 24-well plates containing the medium and cultured for 14 days (Thincert™ PET (Polyethylene Terephthalate) cell culture insert for 24-well plates, 3.0 μ m-pore diameter Greiner Bio-One™, 662631). HepaRG were grown for 7 days in an ECIS plate as described earlier. We designed and 3D printed connectors between the ECIS well and the Transwell insert using the online tool Tinkercad® as illustrated in **Figure 2.5.A**. The HepaRG in the ECIS well were cultured in MMM (viability of Caco-2 cells in MMM was checked

beforehand), bacteria were resuspended in MEME+ and added to the Caco-2 containing insert (at a MOI of 50 corresponding to the HepaRG cells in the ECIS well). The inserts were then connected to the ECIS plate and HepaRG impedance, resistance and capacitance were recorded for 48 hours (**Figure 2.5.B**).

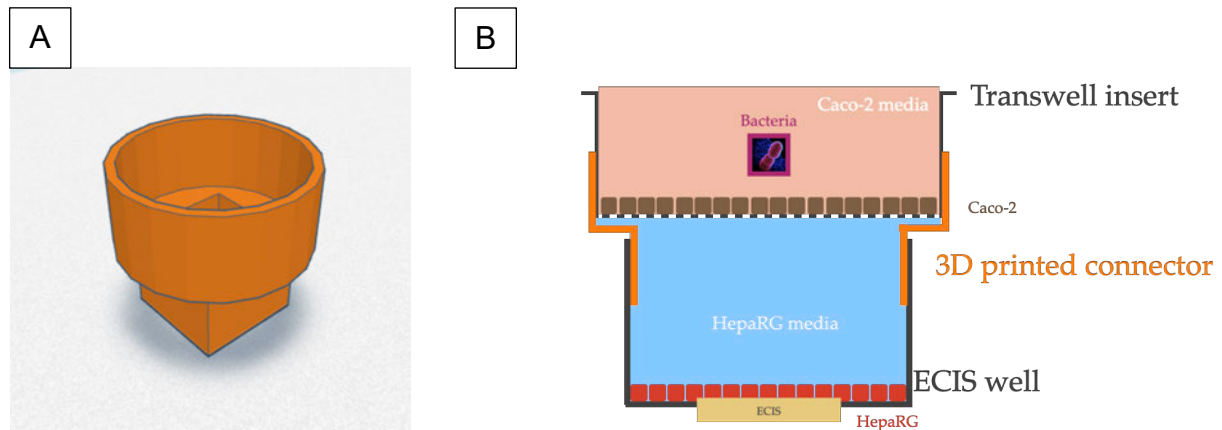


Figure 2.5. A. 3D design of the ECIS – Insert connector. B. Schematic representation of the experimental setup.

2.3.6 Simple Transwell model

Caco-2 cells were seeded in Thincert™ cell culture insert for 24-well plates (Greiner, 3.0 µm-pore diameter – Reference: 662631). They were then infected by our strains at a MOI of 50. The media in the lower chamber was collected every hour for 5 hours and plated using appropriate dilutions to assess the number of bacteria crossing the epithelial barrier over time.

2.3.7 TEER (TransEpithelial Electrical Resistance)

Caco-2 cells were seeded in Thincert™ cell culture inserts for 24-well plates (Greiner, 3.0 µm-pore diameter – Reference: 662631). The media was changed and TEER was measured every 2 days. After 14 days (when a plateau phase was obtained for TEER

values), the TEER between the insert and the media in the lower chamber was measured using a Merck Millicell-ERS2™ Volt-Ohm Meter. Bacteria, resuspended in MEME+, were then added to the cells in the insert at a MOI of 50 at 37°C, 5% CO₂. TEER was recorded every 30 minutes for 5 hours (**Figure 2.6**).

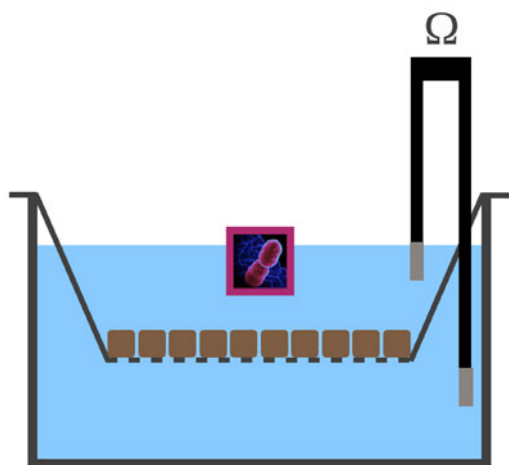


Figure 2.6. Schematic representation of our TEER measurements

2.3.8 PrestoBlue assay

HepaRG cells, seeded in a 96-well plate, were grown for 7 days. They were then infected with bacteria resuspended in MMM without antibiotics for 4 hours at 37°C, 5% CO₂. The supernatant was then discarded, and the wells were washed vigorously. PrestoBlue™ 10% (v/v) (Life Technologies – Reference: A-13262) was diluted 1/10 in PBS with CaCl₂ and MgCl₂. Eukaryotic cells were first washed with PBS with MgCl₂, then 100 µl of the PrestoBlue™ suspension was added in each well of the 96-well plate. After a 30-minute incubation, the reagent was transferred into white walled plates to measure the fluorescence on the GloMax™ (Promega, Southampton, UK) at 571 nm excitation and 584 emission.

2.3.9 LDH secretion measurement

HepaRG cells seeded in 96-well plates were grown for 7 days. They were then infected with 100 µl of bacteria at MOI of 50 resuspended in MMM without antibiotics for 4 hours. We used media only and bacteria only as negative controls. We then used the LDH-Glo™ Cytotoxicity Assay kit (Promega – Reference: J2380), according to the manufacturer's instructions. Briefly, the supernatant was collected and spun down to decrease the bacteria in suspension. 5 µl of the top layer of the centrifuged supernatant was then added to 45 µl of LDH storage buffer, mixed with 50 µl of detection reagent and incubated at room temperature for one hour. The luminescence was then measured using the GloMax™ plate reader.

2.3.10 CellTox™ Green Cytotoxicity Assay

HepaRG cells seeded in a 96-well plate were grown for 7 days. They were then infected with bacteria resuspended in MMM without antibiotics for 4 hours at 37°C, 5% CO₂. The supernatant was then discarded, and the wells were washed vigorously. 100 µl of the CellTox™ Green Reagent (2X, Promega – Reference: G8741) was added to each well, followed by a 15-minute incubation at room temperature. The fluorescence was then recorded (excitation wavelength 485 nm, emission 520 nm) using a GloMax™ plate reader.

2.3.11 Cell viability by flow cytometry

HepaRG were grown in a 48-well plate for 7 days, as described previously. They were then infected for 4 hours at a MOI of 50 at 37°C, 5% CO₂. Triton-X was added to a final concentration of 0.1% for 30 minutes for positive control, and non-infected wells were used as negative controls. After three washes, cells were lifted using TrypLE

(ThermoFisher – Reference: 12604013) and resuspended in PBS complemented with 0.5% FCS. They were then stained with HLA-ABC PE-Cy⁷ (1:1000 dilution, BD Biosciences - Reference: 561349) and Sybr Green (1:1000 dilution, Invitrogen – Reference: S7563) and run through the BD LSR Fortessa™ Cell Analyzer. The data were analysed using FlowJo™ v9.2.

2.4 Innate immune response assessment

2.4.1 Cytokine secretion

The levels of interleukin-8 (IL-8), interleukin-6 (IL-6), interleukin- 10 (IL-10), and tumor necrosis factor (TNF) cytokines in solution were assessed using the BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit™ (BD - Reference: 551811), following the manufacturer's instructions and according to the principle illustrated in **Figure 2.7**. Briefly, Caco-2 and HepaRG cells were grown in 96-well plates, and infected with the different strains at a MOI of 50 for 4 hours at 37°C, 5% CO₂. The supernatant was collected and spun down to pellet bacteria. 50 µl of the supernatant was then mixed with 50 µl of a mix of all 6 cytokine bead solutions previously prepared. This mix was incubated for 3 hours, protected from the light at room temperature. After incubation, the beads were washed with provided Wash Buffer and 50 µl of PE (Phycoerythrin) Detection Reagent added. After washing, the beads were resuspended for data acquisition on a flow cytometer BD LSR Fortessa™ Cell Analyzer. The data were analysed using FlowJo™ v9.2.

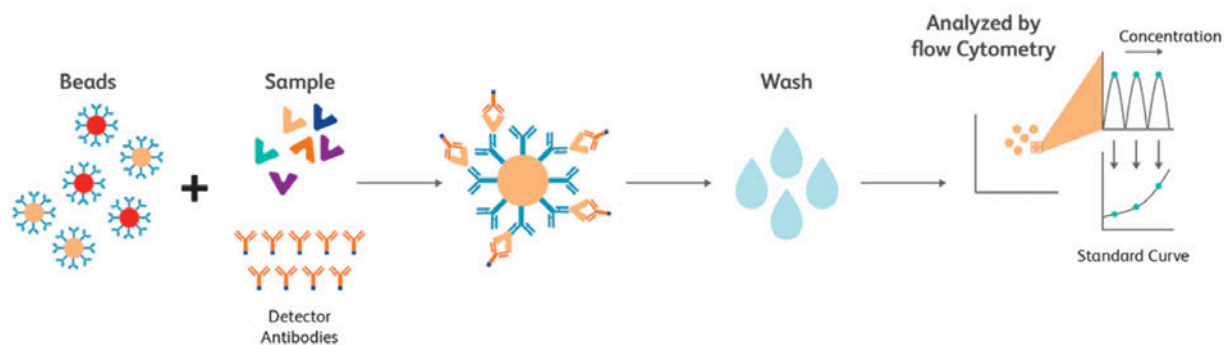


Figure 2.7. Principle of the CBA assay reproduced from the manufacturer's manual.

2.4.2 Chemotaxis assessment using ECIS

2.4.2.1 *Isolation of human peripheral blood mononuclear cells (PBMC) and culturing into monocytes-derived macrophages (MDM)*

The procedure for isolating PBMC from the blood of healthy volunteers was conducted with ethical approval and informed consent. 40 ml of blood was taken using a 21G needle, mixed with 4.4 ml of 3.8% tri-sodium citrate, and centrifuged to separate platelet-rich plasma (350g for 20 minutes, 20°C). This plasma was then treated with CaCl_2 to isolate autologous serum. 6 ml of a 6% dextran solution was used to separate leukocytes from erythrocytes. Next, a three-layer Percoll™ (GE Healthcare - Reference: 17-0891-02) gradient centrifugation process was used to fractionate the leukocytes, isolating PBMC at the 68%/81% interface.

For macrophage cell culture, the isolated PBMC were counted, centrifuged, and suspended in RPMI-1640 media with GlutaMAX™ supplement (ThermoFisher - Reference: 61870010) and 10% FCS. They were then placed in culture plates and incubated. After removing non-adherent cells, the media was replaced every 3 days. Over 14 days, adherent monocytes differentiated into a monolayer of monocyte-derived macrophages (MDM). The maturation of MDM was confirmed through light microscopy, and cell density was assessed after two weeks, showing a consistent number of MDM per well.

2.4.2.2 *Chemotaxis assay using ECIS*

Our experimental setup was inspired by Pietrosimone et al.²⁴⁷ We used special ECIS plates with 8 wells each containing a single linear electrode (Thistle Scientific -

Reference: 8W1CXE). The principle of our assay is illustrated in **Figure 2.8**. 300 μ l of RPMI-1640 media supplemented with 0.5% agarose was added to each well, then allowed to set at room temperature. Once set, we drilled two holes 2 mm apart on either side of the linear electrode using a 14-gauge blunt-end needle. The first of these newly formed wells was filled with MDM, while the second was filled with bacteria (SGH10, SGH10-p and Ecl8), both resuspended in RPMI. The migration of MDM towards the bacteria was quantified by recording the increase in resistance they caused as they passed over the central electrode.

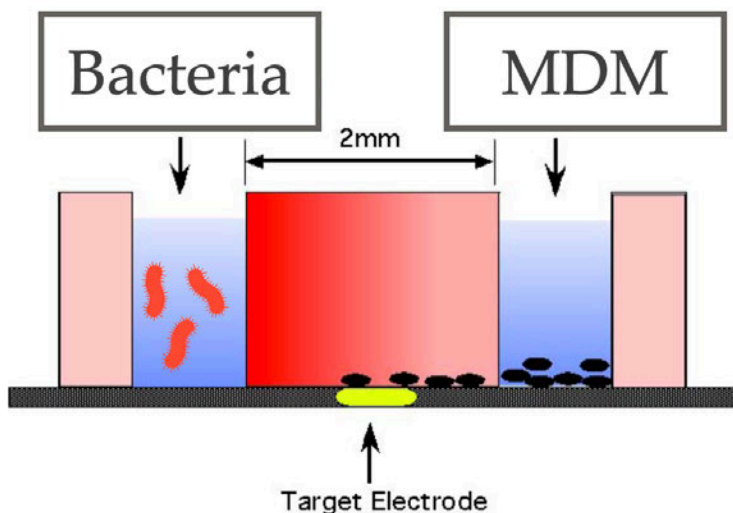


Figure 2.8. Representation of our chemotaxis assay using ECIS. Adapted from ²⁴⁷.

2.5 Western blotting for Caco-2 cells tight junction proteins

Caco-2 cells were seeded in MEME+ and grown for 14 days to ensure fully developed tight junctions. Fresh media containing bacteria at a MOI of 50 was added to the cells, followed by incubation for 4 hours at 37°C. Control wells were treated with EGTA in fresh medium at a final concentration of 1 mM for the last 30 minutes of incubation. The medium was aspirated from the cultures, and the cells were lysed with MRC PPU lysis buffer (composition summarised in **Table 2.8**, 1 volume per well of a 96-well plate), containing 1/25 volume of Protease inhibitor 25x, 3 µl/ml of Benzonase, and a final concentration of 2 mM MgCl₂. The protein concentration was quantified using the BCA (Bicinchoninic acid assay) protein assay kit (Pierce, ThermoFisher) according to the manufacturer's instructions. 10 µl of lysate containing 20 µg of protein was added per well onto an SDS-PAGE gel (Stacking: 6% Acrylamide, Running: 15% Acrylamide), at 75 volts (V) for 30 minutes, followed by 60 minutes at 135 V.

The proteins were electro-transferred to a nitrocellulose or PVDF membrane in a semi-dry condition at 20 V for 90 minutes. The nitrocellulose membrane was incubated in 25 ml of blocking buffer (5% skimmed milk in 0.2% TBST) for 1 hour at room temperature, and then incubated with primary antibodies diluted (1/1000) in TBST with 5% skimmed milk at 4°C with gentle shaking overnight. Primary antibodies specific for ZO-1 (ThermoFisher – Reference: 40-2200), claudin-1 (ThermoFisher – Reference: 51-9000), claudin-3 (ThermoFisher – Reference: 34-1700) and occludin (ThermoFisher – Reference: 33-1500). After thorough washing, the membrane was incubated with anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology – Reference: #7074) at a 1:3000 dilution in 10 ml of blocking buffer with gentle agitation

for 1 hour at room temperature. SuperSignal™ West Pico PLUS Chemiluminescent Substrate was added and incubated for 5 minutes, before detection.

<u>Final conc.</u>	<u>Chemical</u>	<u>Stock</u>	<u>Quantity</u>
50 mM	Tris pH 7.5	1M Tris-HCl pH7.5	25 ml
1 mM	EDTA	0.5M EDTA pH8.0	1 ml
1 mM	EGTA	0.5M EGTA pH8.0	1 ml
10 mM	glycerol 2-phosphate	200mM	25 ml
50 mM	NaF	500mM	50ml
5 mM	Na pyrophosphate	50mM	50ml
270 mM	sucrose (saccharose)	powder	46.21g
1%	Triton	100%	5ml
	H2O		till 500ml

Table 2.8. Composition of MRC PPU lysis buffer

2.6 Zebrafish infection

2.6.1 Collection and preparation of zebrafish embryos

The embryos were collected from the University of Edinburgh aquatics facility (Wick line, QMRI Aquatics Facility) and transported in a Petri dish containing around 15 ml of E3 media (see **Table 2.9**), lid taped down to reduce spillage and in a suitable container. Embryos were then placed in a 28°C incubator. After 2-3 hours, they were transferred into a Petri dish containing conditioned water from the aquatics facility and supplemented with 0.2 mM PTU (Phenylthiourea) to reduce melanisation. The dishes were then examined by microscopy to remove any non-fertilized eggs and debris left by adult fish and fungi. The embryos were then divided into groups of 50, each group transferred into fresh E3 medium in a new Petri dish and incubated at 28°C. After 3 days of incubation, the embryos were taken out of the incubator, and released from their envelope using tweezers under the microscope. They were then ready for use.

Chemical/Solute	Quantity
NaCl	34.8 g
KCl	1.6 g
CaCl ₂ ·2H ₂ O	5.8 g
MgCl ₂ ·6H ₂ O	9.78 g
H ₂ O	2 L
Methylene blue	Added 100 µl to the 1X media

Table 2.9. Composition of E3 media (60X stock)

2.6.2 Infection by immersion

Bacterial strains were grown in LB broth at 37°C overnight. The next morning, they were subcultured 1:100 into fresh LB and grown for 3 hours at 37°C, shaking, to reach an $OD_{600} \sim 1$, approximatively corresponding to 10^9 cfu/ml. This culture was then centrifuged and resuspended in the same volume of E3 media to give a concentration of 10^9 cfu/ml. 20 ml of this mix was added to a Petri dish with 20 zebrafish larvae. The Petri dish was then placed in a 28°C incubator.

2.6.2.1 *Survival rate*

The number of live embryos was determined visually using a microscope after 12, 24 and 48 hours and plotted on a survival curve.

2.6.2.2 *Dissemination rates*

After 24 hours, zebrafish embryos were collected, washed twice in E3, resuspended in E3 containing tricaine for anaesthesia and crushed in the bottom of a 1.5 ml Eppendorf. After centrifugation, the pellets were resuspended in PBS and plated on LB agar plates with appropriate dilutions.

2.6.2.3 *Imaging*

After 24 hours incubation with bacteria, zebrafish embryos were collected, washed twice in E3, resuspended in E3 containing for anaesthesia and finally mounted on coverslips, in 1% low melting point agarose (Sigma-Aldrich – Reference: 9012-36-6), for confocal microscopy.

2.7 Statistical analysis

Most statistical analyses and graphs were prepared using both Microsoft Excel 2020 and GraphPad Prism 9. For simple comparisons, the statistical tests used were either one-way ANOVA tests comparing variances or t-tests comparing the means, where appropriate. The threshold of significance was set at $p < 0.05$. For the comparison of more than one sample and the measurement of the collection of data over time within an experiment, two-way ANOVA with Sidak's multiple comparisons test were performed. Rstudio was used to compute a script to calculate the T_{50} decrease factor using ECIS. TEER results were processed using mixed effects models to avoid auto-correlation.

Chapter 3: Characterisation of *Kp*'s interactions with

eukaryotic cells and role of the virulence plasmid

3.1 Introduction

There are no clear and defined criteria for describing a strain of *Kp* as hypervirulent. Ideally, this should be based on the combination of genotypic, phenotypic and clinical features.^{110,248} The main microbiological conditions are a thick and viscous capsule, siderophores and colibactin secretion. These characteristics stem from the presence and expression of the genes coding for them (summarised in **Table 3.1**): hypercapsule through *rmpA(2)*, *rmpC*, *kvrA/B*, *rscA/B*; hypermucoviscosity through *rmpD* and *rmpA(2)*; aerobactin through *iuc*; yersiniabactin through *ybt*; salmochelin through *iro* and genotoxin colibactin through *clb*. However, these regulators can also be found in non-Hv*Kp* strains.^{81,83} It is thus unclear if they are differentially expressed or if the conjunction of factors eventually makes a strain hypervirulent. These microbiological criteria must be combined with a clinical virulence criterion, i.e for Hv*Kp* the ability to cause severe infections in young and immunocompetent patients.

To distinguish the role of the various major virulence factors, we chose to use SGH10, described as the archetypal Hv*Kp* strain by phylogenetic analyses in 2018.⁸³ SGH10, K1 serotype, has *ybt*, *clb* and *iro* integrated to its 5.4Mb chromosome, but also possesses a 2.3Mb virulence plasmid (pSGH10) carrying *iro*, *iuc*, *rmpA(2)*, *rmpC* and *rmpD*. This pSGH10 has been shown to be highly comparable to the 2 main virulence plasmids described to date (pK2044 and pLVPK)²⁴⁹, and is crucial for virulence of Hv*Kp* in *in vivo* models.^{250–252} SGH10 also exhibits central phenotypes of Hv*Kp*, i.e.

hypermucoviscosity and an hypercapsule providing resistance to opsonisation and phagocytosis.²⁵³

To establish how the genes carried on the virulence plasmid were relevant to HvKp's physiopathology, we generated a strain lacking this virulence plasmid, that we called SGH10-p. The absence of the virulence plasmid results in the loss of both the genes coding for the hypercapsule/HMV phenotype (*rmpA/C/D* locus) but also the siderophore aerobactin (*iuc*, main siderophore associated with virulence¹¹¹). We thus needed a third strain to untangle the specific role of each of those two major virulence factors.

We chose Ecl8, a K2 capsular serotype strain belonging to the ST375 of the hypervirulent clonal group CG65 which has a virulence plasmid encoding aerobactin, salmochelin and the regulator of mucoid phenotype, *rmpA2*.²⁵⁴ Yet, interestingly, whilst the Ecl8 *cps* locus shares 98.4% nucleotide identity with the widely studied hypercapsulated and hypermucoviscous KPRR1 strain K2-capsule loci, the region downstream of *rmpA2* only shares 92.6% nucleotide identity with the sequence from KPRR1 (unpublished data). This is expected to lead to a frameshift and potential loss of function of *rmpA2*, *rmpD* and *rmpC*. In fact, Ecl8 had been previously shown as having normal capsule levels.²⁵⁵

In summary, as summarised in **Table 3.1**, SGH10 should secrete aerobactin and be hypercapsulated/HMV, SGH10-p should lose aerobactin secretion and its hypercapsulation/HMV phenotype, whilst Ecl8 should still secrete aerobactin but also have basal capsulation and mucoviscosity levels.

	Genes coding for capsule				Genes coding for HMV		Gene coding for siderophores					Expected phenotype			
	<i>rmpA(2)</i>	<i>rmpC</i>	<i>kvrA/B</i>	<i>rcaA/B</i>	<i>rmpA(2)</i>	<i>rmpD</i>	<i>iuc</i> (aerobactin)	<i>iro</i> (salmochelin)	<i>ybt</i> (yersinibactin)	<i>ent</i> (enterobactin)	Hyper capsule	HMV	Aerobactin secretion		
SGH10	+	+	+	+	+	+	+	+	+	+	+	+	+		
SGH10-p	-	-	+	+	-	-	-	-	+	+	-	-	-		
Ecl8	?	?	+	+	?	?	+	+	+	+	-	-	+		

Table 3.1. Summary of the presence of the main genes responsible for hypercapsule, HMV and siderophores secretion in SGH10, SGH10-p and Ecl8, and the resulting expected phenotype.

+: Present or expected phenotype.

-: Non-present, or unexpected phenotype.

?: Gene present but probably non-functional.

Overall, the aim in this chapter was therefore to characterise our strains and ascertain that they present with different and distinctive hypervirulent features. This would make them relevant for the development of a model to study the role of these hypervirulent features in *HvKp*'s pathogenicity in the human host.

3.2 Results

We compared our strains first in terms of growth (especially in tissue culture media, as this could bias future *in vitro* infection assays), but more importantly in terms of capsulation/viscosity levels and siderophore production. We then measured their hypervirulence and capsule gene expression by qPCR. Subsequently, we compared the 'simple' interactions with gut, liver and endothelial cells, as one of HvKp's main traits is the low adhesion and internalisation to/in epithelial cells.^{190–194} These assays were carried out by infecting the 3 cell types encountered in the liver abscess formation process: intestinal (Caco-2), endothelial (HUVECS) and hepatic (HepaRG) cell lines. We next looked at the interactions of our strains with the innate immune system cells (monocytes), as this ability to evade immune system is also paramount for HvKp's pathogenicity.²⁵⁶ Our final validation of potential differences in virulence involved an immersion infection model of zebrafish larvae, as this has already been shown as accurately discriminating virulence of Kp.²³⁸

3.2.1 Growth curves of bacteria

3.2.1.1 *Growth of the strains in LB broth and eukaryotic tissue culture media*

As our infection assays use eukaryotic cells, it was crucial to ensure that bacterial cell growth was not impacted in tissue culture media. We assessed the growth of our strains in Luria-Bertani (LB) media, PBS, MEME+ (media for Caco-2) and MMM (media for HepaRG cells). As illustrated in **Figure 3.1**, showing the OD₆₀₀ over time, our strains didn't grow in PBS, their growth was comparable in LB, but SGH10 and SGH10-p grew more slowly than Ecl8 in TC media. In LB, all strains reached an OD₆₀₀ of 1 and entered in a stationary phase within approximately the same timeframe. When looking at

MEME+ and MMM media, whilst all strains did grow, SGH10 and SGH10-p grew more slowly than Ecl8. SGH10 and SGH10-p only reached an OD₆₀₀ of 0.75 in MEME+ and 1 in MMM after 5 hours of incubation, compared with 2 and 2.5, respectively, for Ecl8. This seemed to indicate that the TC media were less suitable for our SGH10 and SGH10-p. To note, as SGH10 and SGH10-p are sensitive to streptomycin, it was omitted from HepaRG MMM media (MIC < 2, data not shown)

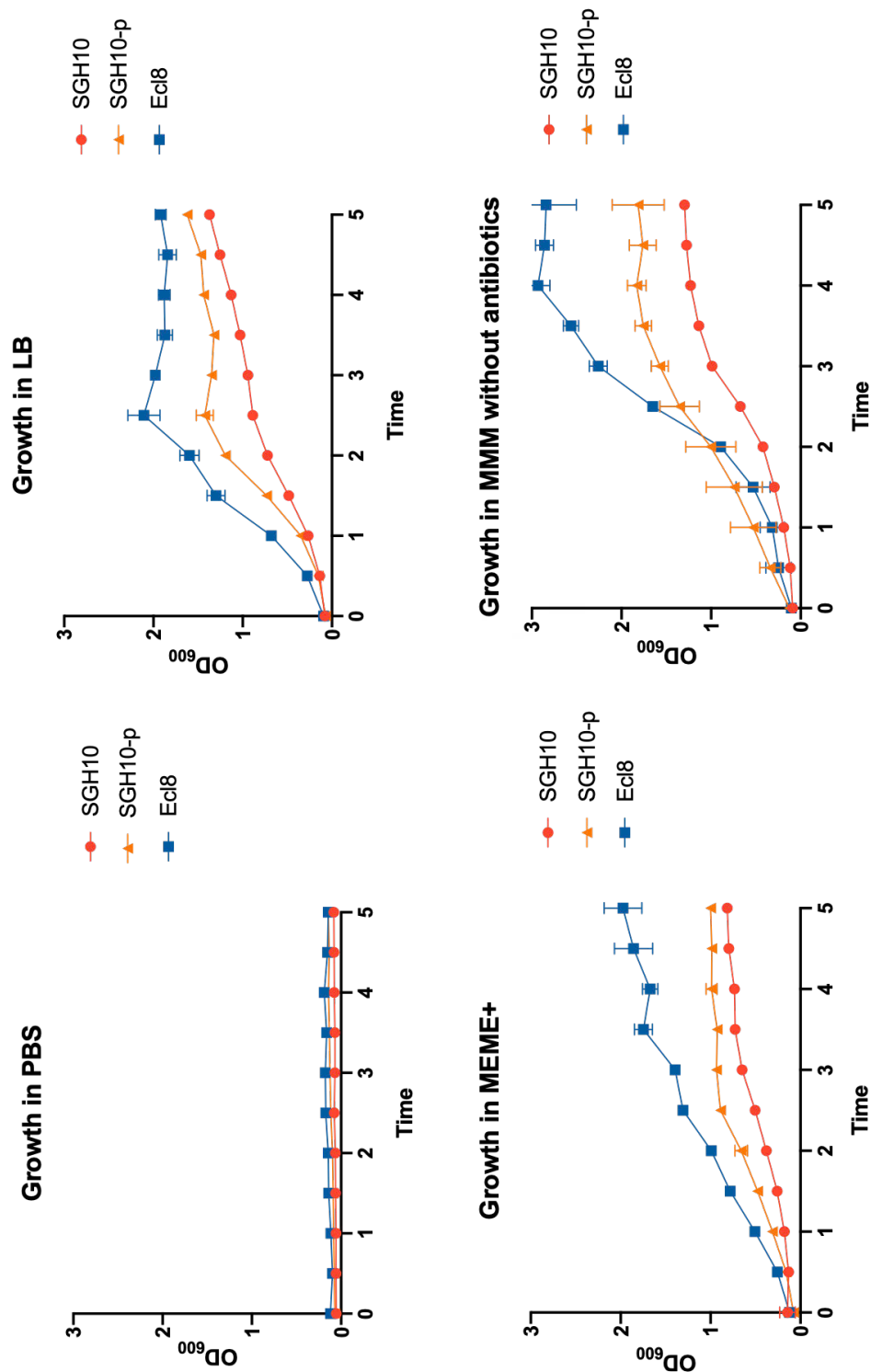


Figure 3.1. Growth of SGH10, SGH10-p and Ecl8 in PBS, LB, MEME+ (Caco-2 media) and MMM (HepaRG media).

Results expressed as OD₆₀₀ over time (recorded over a period of 5 hours).

Single biological but triple technical replicates.

3.2.1.2 Relationship between optical density (OD) and bacterial growth (cfu/ml)

The accurate determination and standardisation of the number of bacteria applied to eukaryotic cells in further infection assays was crucial. This was ensured by establishing the relationship between the OD₆₀₀ of our bacteria grown in LB and the number of cfu (obtained after plating appropriate dilutions). **Figure 3.2**, plotting the OD₆₀₀ as a function of cfu/ml for SGH10, SGH10- and Ecl8, shows a sigmoidal relationship between the OD₆₀₀ and the concentration (cfu/ml) of bacteria, comparable for all three strains. These curves were subsequently used to accurately extrapolate the number of cfu before infection.

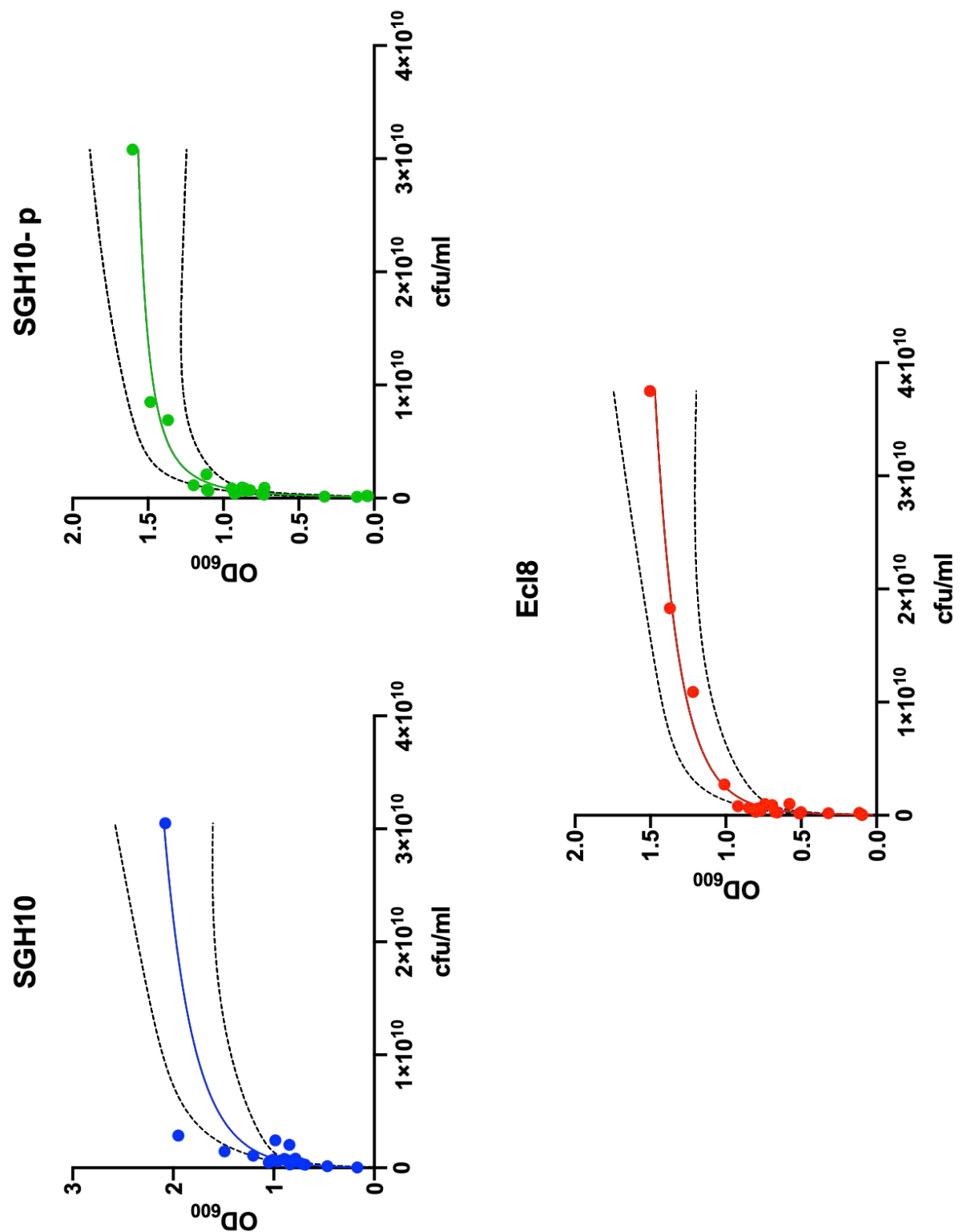


Figure 3.2. Relation between OD and cfu/ml for SGH10, SGH10-p and Ecl8 grown in LB broth, plotted with 95% confidence interval.

Each point representing one biological replicate.

3.2.2 Capsule production and hypermucoviscosity

To establish the differential levels of capsule production and HMV, we used glucuronic acid production quantification and low-speed centrifugation, respectively.

The level of capsule production was first assessed by applying a percoll gradient and measuring the glucuronic acid production, extracted using the Zwittergent method. The percoll gradient (**Figure 3.3**) showed that SGH10 and SGH10-p had approximately the same amount of capsule, as they are at the same level when centrifuged. Ecl8 was found at the bottom of the tube, indicating lower capsule production.

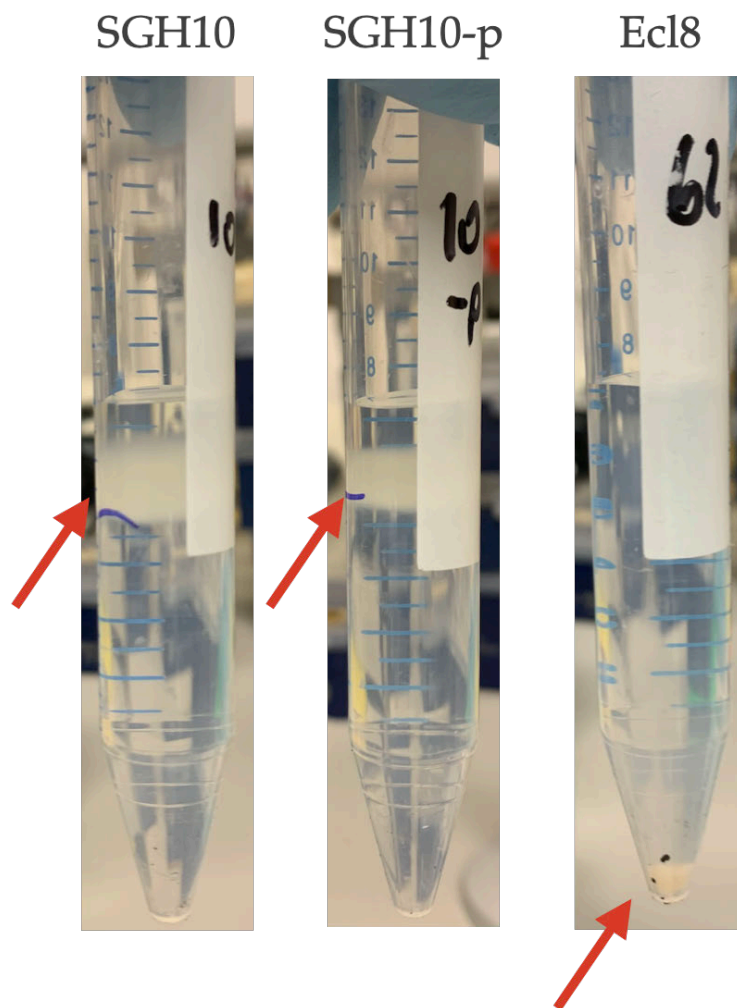


Figure 3.3. Illustration of the Percoll Gradient applied to SGH10, SGH10-p and Ecl8. Red arrows indicate bacteria.

This quantitative assessment was confirmed by glucuronic acid quantification. As shown in **Figure 3.4A**, Ecl8 produced significantly less capsule than SGH10, with a more than 2-fold lower glucuronic acid concentration than SGH10. Whilst SGH10-p did produce slightly less capsule than SGH10 (around 1.3-fold decrease), this was not statistically significant.

We then quantified the viscosity of our colonies using low-speed centrifugation. This assay, described in section 2.1.9, is based on the fact that mucoviscosity proportionally impedes the strain's ability to pellet. While they all presented with significantly variable levels of mucoviscosity and there is no clear-cut value to define HMV, SGH10 was significantly more mucoviscous than both SGH10-p (1.5-fold higher) and Ecl8 (2-fold higher). Yet, SGH10-p preserved a higher mucoviscosity than Ecl8 (1.5-fold higher, see **figure 3.4B**).

Therefore, we concluded that SGH10 and SGH10-p had a slight fitness disadvantage in TC media (growing slightly slower than Ecl8), SGH10 was both HMV and hypercapsulated, while SGH10-p was primarily hypercapsulated and Ecl8 had lower capsule and viscosity levels (summarised in **Table 3.2**, on page 109). Of note, this conservation of a high level of capsulation and low levels of HMV of SGH10-p was unexpected, as it has lost its virulence plasmid which carries most of the genes responsible for both these phenotypes.

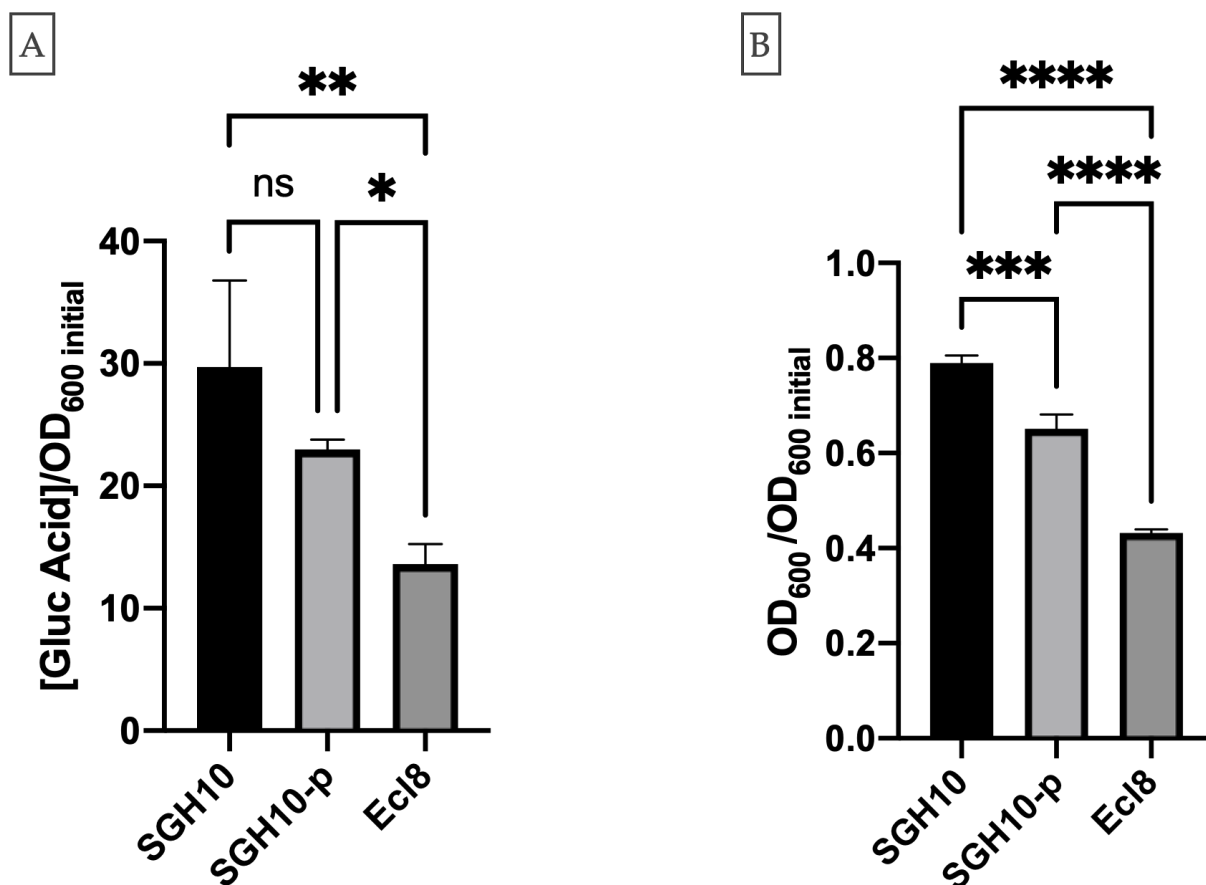


Figure 3.4. Capsule and mucoviscosity measurements.

A. Capsule levels as measured by glucuronic acid concentration (in $\mu\text{g/ml}$) normalised to the OD₆₀₀ of the initial culture. B. Mucoviscosity of the strains expressed as OD₆₀₀ of the supernatant after low-speed centrifugation, normalised to the OD₆₀₀ of the initial overnight culture. Bars represent mean and standard deviation of three technical and biological replicates, compared with a one-way ANOVA test with multiple comparisons. ns = non-significant. *adjusted $p < 0.05$. **adjusted $p < 0.01$. ***adjusted $p < 0.001$. ****adjusted $p < 0.0001$.

3.2.3 Siderophores production assessment using CAS plates

We next assessed the levels of siderophores production using CAS plates (described in section 2.1.10). These plates appear blue following incorporation of the iron (part of the Fe-CAS-hexadecyltrimethylammonium complex) into the agar. Upon siderophores

production, the iron is captured, which will lead to a change of colour to orange. **Figure 3.5A** shows that colour change following incubation of the three strains. The diameter of that colour change was measured, and **Figure 3.5B** shows the results of three biological replicates. This shows SGH10 and Ecl8, both carrying *iuc* coding for aerobactin, had comparable siderophore secretion, which was significantly higher than SGH10-p, which has lost the *iuc* gene upon loss of plasmid.



B

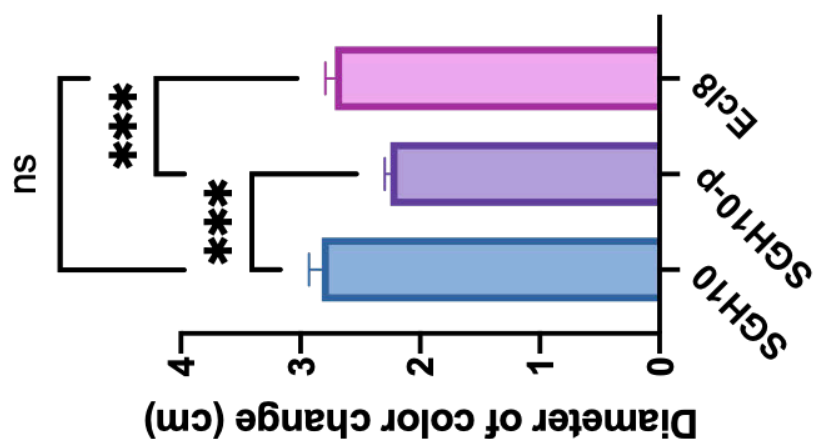


Figure 3.5. A. CAS plate with measure of the diameter of the color change induced by SGH10, SGH10-p and Ecl8. B. Graphs of the diameter of the color change of three biological replicates.

Bars represent mean and standard deviation of three technical and biological replicates, compared with a one-way ANOVA test with multiple comparisons. ns = non-significant.

***adjusted $p < 0.001$.

3.2.4 qPCR for *rmpA/C*, *iuc* and capsule genes *wzi* and *wcaJ*

To confirm and elucidate the phenotypes observed in terms of capsulation, mucoviscosity and secretion of siderophores, we studied the expression of the genes responsible for these via qPCR. We looked at the genes encoding HMV (*rmpA*), hypercapsule (*rmpC*), aerobactin (*iuc*) or being part of the *cps* locus (*wzi* and *wcaJ*). The results are shown in **Figure 3.6**.

rmpA expression was increased in SGH10 compared to SGH10-p, as expected. However, we thought that the frameshift present in Ecl8 would result in suppressing its expression, which was not the case: Ecl 8 expressed *rmpA* around two times less than in SGH10, but still almost 30 times more than SGH10-p.

The exact same trend was observed with *rmpC*. This was unexpected given the total absence of HMV and hypercapsule in Ecl8.

We thus looked at the expression of two genes at either end of the *cps* locus, *wcaJ* (which initiates capsule formation by attaching the first glucose to a phosphate) and *wzi* (which anchors capsule to the surface of the bacteria). Very surprisingly, *wcaJ* expression in SGH10 was much lower than in the 2 other strains. Yet, it was more expressed in SGH10-p than in Ecl8, potentially indicating a decrease in its absolute expression.

The expression of *wzi* was much more consistent with our strains' observed phenotype, with no difference between SGH10 and SGH10-p, but a 10-fold lower expression in Ecl8, that could explain this low capsulation.

Finally, *iuc* expression, representing the siderophore aerobactin, was also in line with the CAS plate results, with a preserved and comparable expression in SGH10 and Ecl8, but much decreased in SGH10-p.

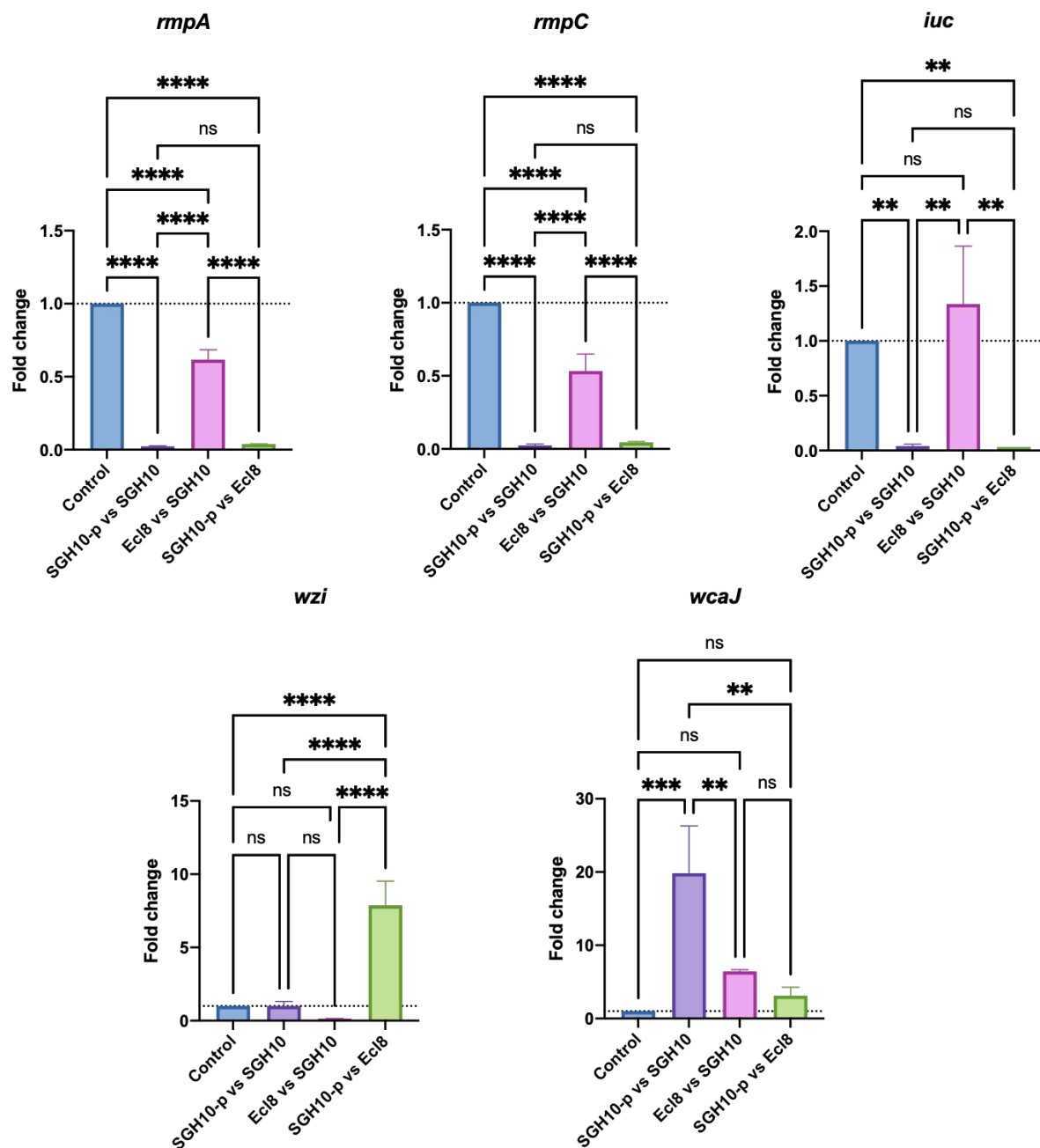


Figure 3.6. Pairwise comparison of virulence and capsule genes expression fold change between SGH10, SGH10-p and Ecl8.

“Theoretical” control = 1 (no change). Bars represent mean and standard deviation of three technical and biological replicates, compared with a one-way ANOVA test with multiple comparisons. ns = non-significant. *adjusted $p < 0.05$. **adjusted $p < 0.01$. ***adjusted $p < 0.001$. ****adjusted $p < 0.0001$.

The main characteristics of the three strains, compared with the genes coding for virulence factors (including *rmpA(2)*, *rmpC* and *rmpD*, coding for HMV and hypercapsule) that were functional, are listed in **Table 3.2**.

Strain	K serotype – Clonal Group	Virulence plasmid	Functional main genes coding for virulence factors										Phenotype			
			rmpA	rmpA2	rmpC	rmpD	iro	iuc	ybt	ent	clb	Capsule	HMV	Sideropho res		
SGH10	K1 – CG23	+	V	V	V	V	V	V	V	V	V	-	-	-		
SGH10-p	K1 – CG23	-	X	X	X	X	X	X	X	V	V	-	-	N		
Ecl8	K2 – CG65	+	V	V	V	?	V	V	V	V	X	N	N	-		

Table 3.2. Main genotypical and phenotypical characteristics of the three strains used in our study.

Plasmids: +: present. -: absent.

Genes: V = present and functional. X = absent or non-functional.

Signs in bold and red = expression levels confirmed by qPCR

Phenotype: - = Increase. -- = Major increase. N = Basal levels.

3.2.5 Eukaryotic cells count for determination of the MOI

Having determined the characteristics and growth dynamics of our bacteria, it was important to determine the number of eukaryotic cells present on the plates just prior to their infection, ensuring the most exact MOI possible.

This count was performed using two separate techniques to increase precision, i.e. “visual” using DAPI nuclear staining for automated counting by microscopy using EVOS and by flow cytometry analysis.

As shown in **Table 3.3**, the number of cells counted depended on both the type of cell and the counting technique, with HepaRG cell counts before use (day 7) significantly lower than Caco-2 cells (day 10-14).

The number of cells counted with flow cytometry was generally lower than with visual automated counting with EVOS. Because flow cytometry showed some dead and aggregated cells, likely due to the handling process, we assumed that this technique provided an underestimation of the number of cells in the well. On the other hand, because DAPI would stain nuclei of both live and dead cells, we assumed that visual automated counting with EVOS was overestimating the number of cells in the well. As these numbers were not too far apart, we calculated the average of the 2 techniques to obtain the most accurate number possible.

	HepaRG		Caco-2	
	96W plate	ECIS	96W plate	ECIS
Flow cytometry	30.000	80.000	90.000	240.000
Visual - EVOS	40.000	107.000	130.000	350.000
Mean	35.000	95.000	110.000	300.000

Table 3.3. Average number of HepaRG and Caco-2 counted with Flow-cytometry and visual methods, on a 96-wells plate (0.3cm²) and on ECIS plate (0.8cm²).

N=3 Note: The increased numbers in ECIS as compared to 96 well are due to difference in area as the density of cells is the same. Their growth in both supports is therefore comparable.

3.2.6 Adhesion and internalisation of our strains to intestinal, hepatic and endothelial cells

To establish how our microbial panel would interact in vivo, we undertook adhesion and internalisation assays, as these would establish the basic interactions of bacteria with eukaryotic cells. Performing the assay with bacteria resuspended in TC media, made exact determination of the inoculum unpredictable as the bacteria continued to grow in it. This was also observed despite lower levels of fetal calf serum. We therefore decided to suspend the bacteria in PBS, which eliminated the growth, and produced more reliable results.

The adherence of SGH10 to gut, liver and endothelial cells was lower than SGH10-p and Ecl8 (**Figures 3.7A, C, D**), with on average at least ten times fewer bacteria retrieved after incubation and washing of eukaryotic cells.

Interestingly, all three strains were hardly internalised in both HepaRG and HUVECS, irrespective of their capsulation, HMV or adherence levels. In Caco-2, SGH10 was significantly less internalised than either SGH10-p or Ecl8, even after normalisation for adhesion (**Figure 3.7B**).

This established that both capsulation and HMV were crucial for adhesion to eukaryotic cells, but only HMV seemed to be impeding the phagocytosis of bacteria by intestinal cells.

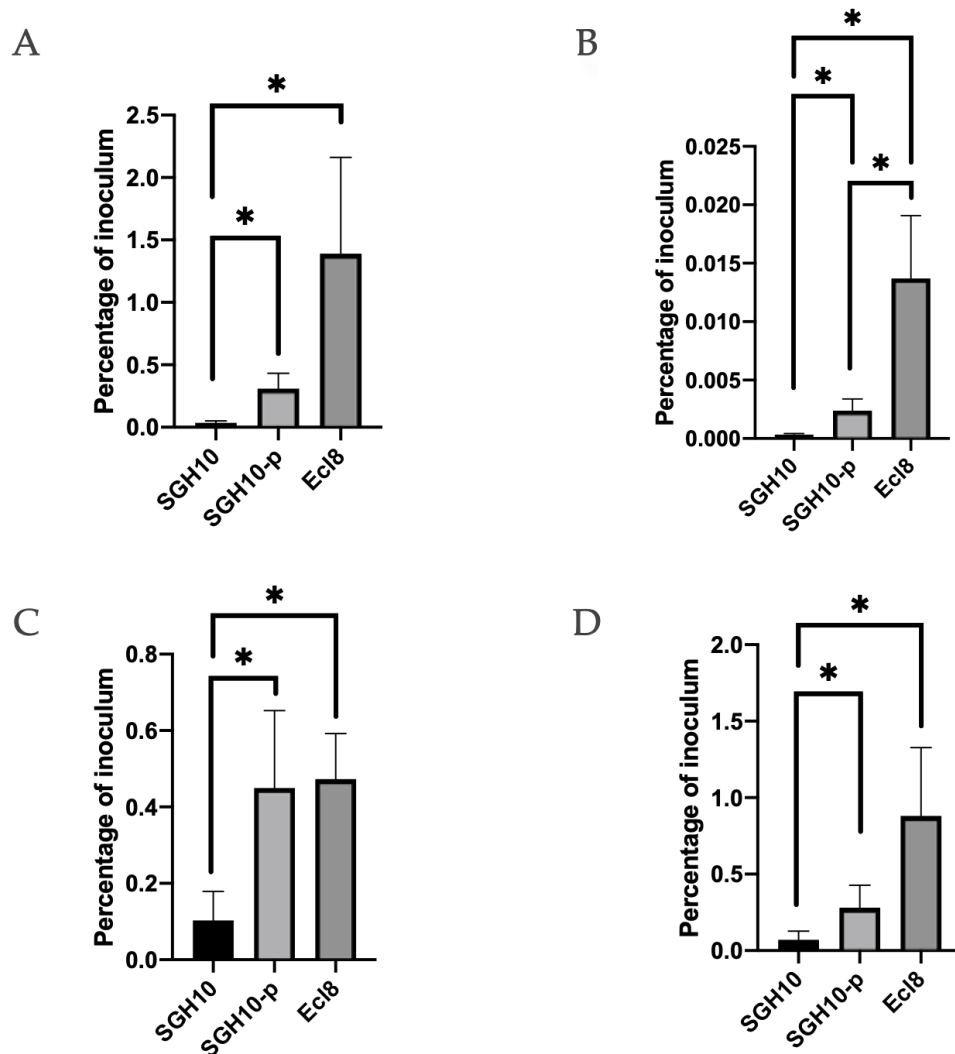


Figure 3.7. Adhesion and internalisation of SGH10, SGH10- and Ecl8 to gut (Caco-2), liver (HepaRG) and endothelial (HUVECS) cell lines.

A. Adhesion to Caco-2 cells B. Internalisation in Caco-2 cells C. Adhesion to HepaRG D. Adhesion to HUVECS. Bars represent mean and standard deviation of three technical and biological replicates, compared with a one-way ANOVA test with multiple comparisons. ns = non-significant. *adjusted $p < 0.05$. Non-significant statistical tests not reproduced on the figure.

3.2.7 Qualitative visualisation of adhesion to eukaryotic cells using immunohistochemistry

To confirm the data obtained in our adhesion assays described above, we attempted to visualise these interactions using an optical microscopy technique. This involved firstly making our bacteria fluorescent by introducing a carrying a green fluorescent protein under a constitutive promoter (pRSMGFP+), and then staining the infected cells.

3.2.7.1 *Electroporation of pRSMGFP+ in SGH10:*

Electroporation of plasmid pRSMGFP+ into SGH10 was initially unsuccessful with both classical electroporation (used for normally capsulated strains) and transconjugation with *E. coli* S17 λ pir. After adaptation of our electroporation protocol for mucoidal cells (as discussed in chapter 2), we finally managed to insert the plasmid, as illustrated in **Figure 3.8**, showing green fluorescent SGH10 .

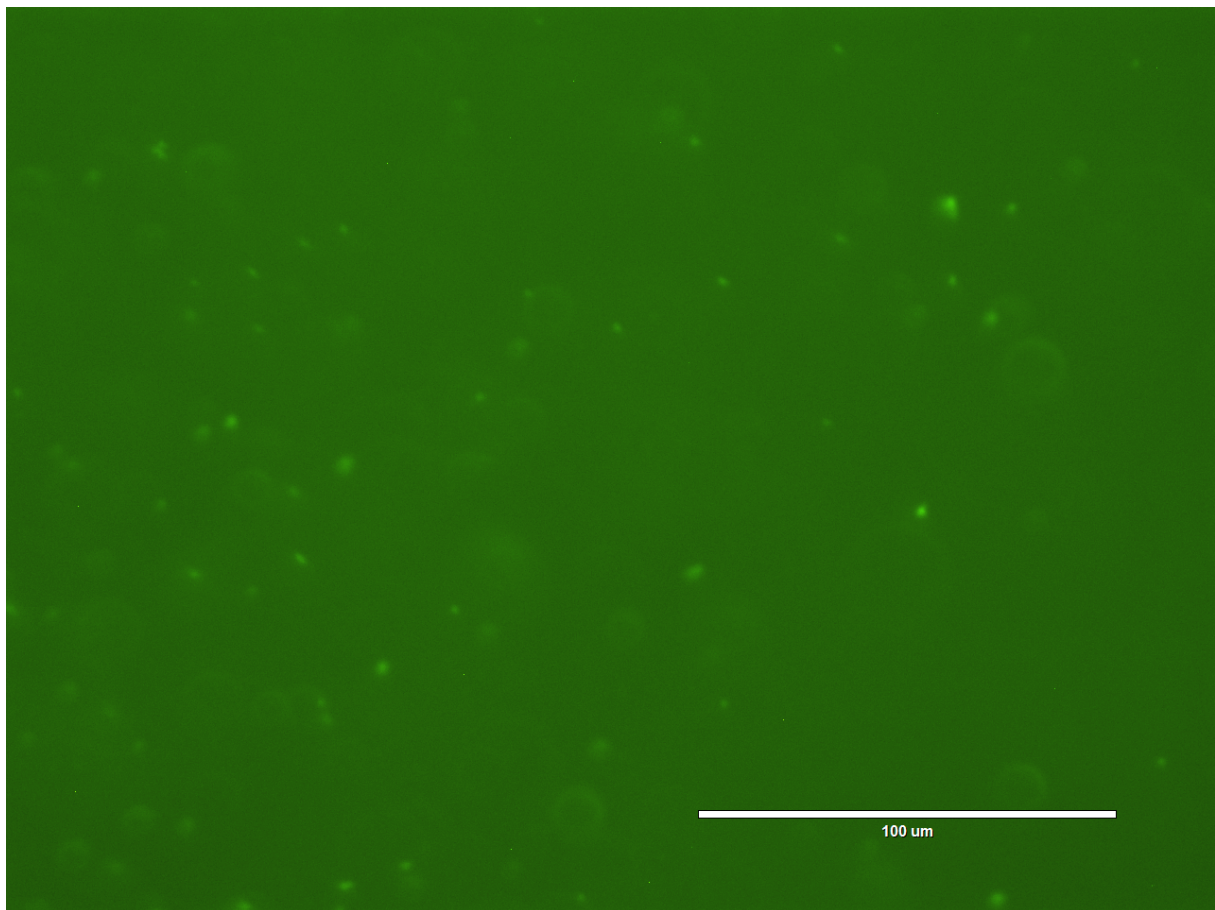


Figure 3.8. GFP-Tagged SGH10 bacteria. 10^4 dilution of an overnight culture.

3.2.7.2 Visualisation of images

We used the EVOS™ microscope to visualise the infection of both Caco-2 and HepaRG cells with our GFP-tagged bacteria. We could not visualise these interactions with Caco-2 cells, as these cells didn't resist formaldehyde fixation and washes, necessary to remove non-adherent bacteria. **Figure 3.9** therefore shows the interactions between fluorescent bacteria (Ecl8 and SGH10) and HepaRG. We could visually see that for the same MOI, more Ecl8 are observed at the surface of eukaryotic cells than SGH10. Also, Ecl8 is more clearly adherent to the cells (at the edge of them), while the few SGH10 found are present in areas free of liver cells.

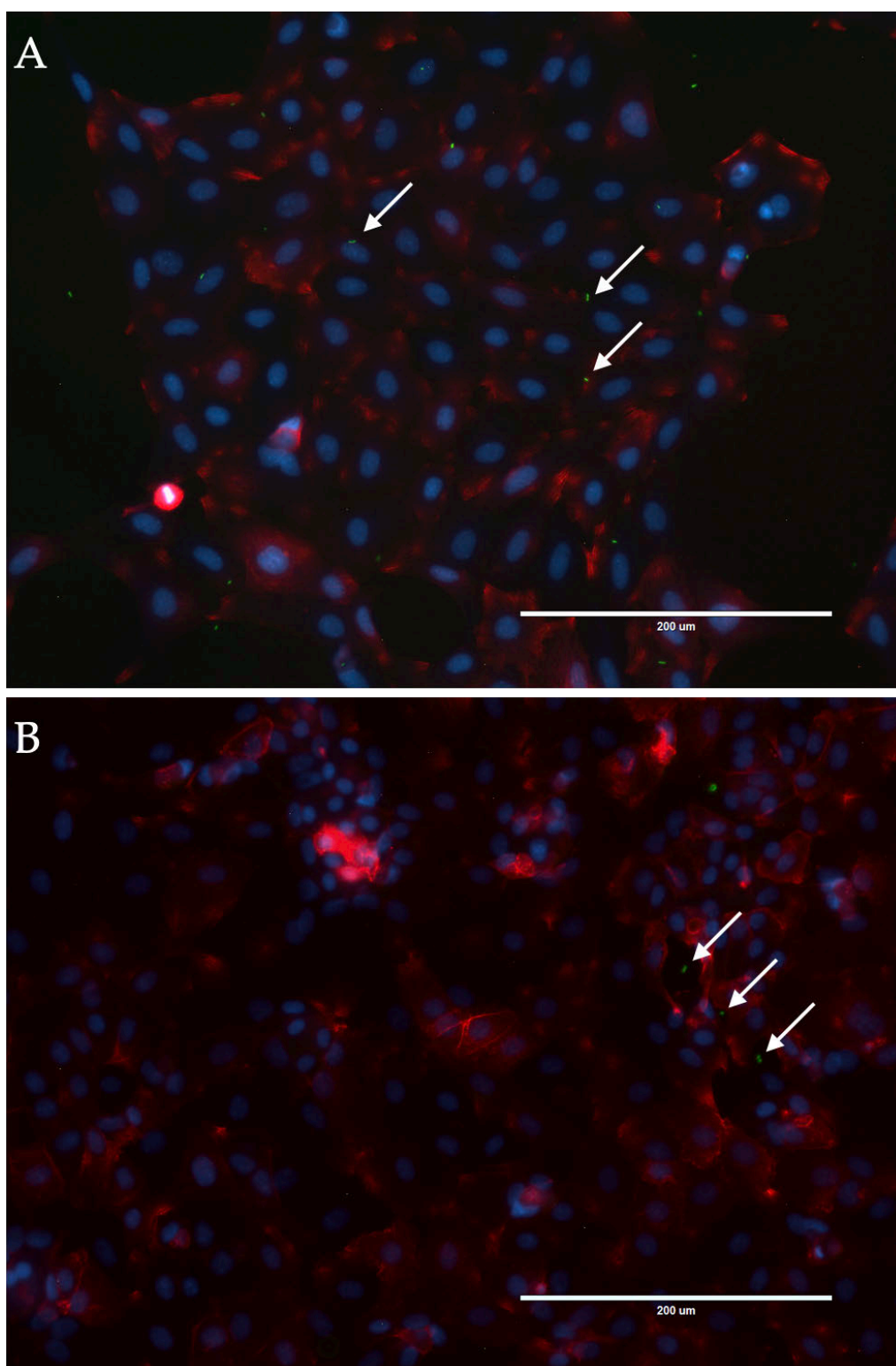


Figure 3.9. HepaRG cells images acquired on EVOS after infection with bacteria. Stained with phalloidin (Red, cytoskeleton of eukaryotic cells) and DAPI (Blue, nucleus

of eukaryotic cells). Green bacteria indicated by white arrows (A) Infected with Ecl8 + pRSMGFP+ strain. (B) Infected with SGH10 + pRSMGFP+.

3.2.8 Interactions with human Monocyte-Derived Macrophages (MDM) using the ECIS system

One of the main characteristics of *HvKp* is the avoidance of the immune system, thought to be linked to their thick capsule and HMV. We thus attempted to measure the chemotaxis of MDMs in response to our different strains, to allow further phenotypic confirmation of their difference of virulence levels. As illustrated in **Figure 3.10**, we found that the level of capsulation (and not HMV) of our strains correlated inversely with MDM migration, with no increase of resistance measured by ECIS for either SGH10 or SGH10-p (close to the media only control line). In contrast, Ecl8 clearly caused MDM migration, as shown by the increase in resistance recorded by the central electrode. For practical reasons (unavailability of these particular ECIS plates from our supplier), we were only able to carry out one biological replicate, and therefore no statistical test was carried out.

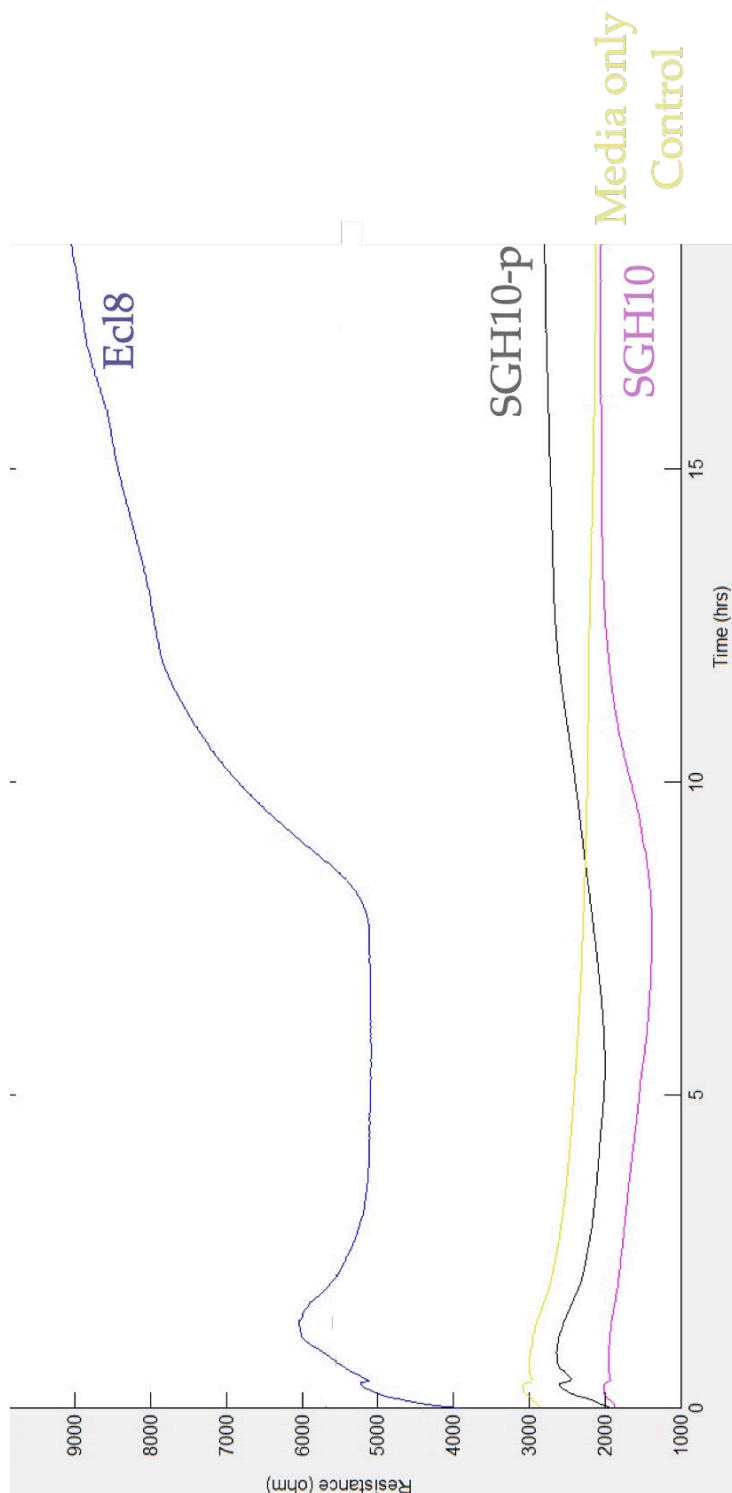


Figure 3.10. Resistance generated by MDM migrating towards the different bacterial strains, recorded by ECIS.

Single biological and technical replicate.

Dark blue line: Ecl8. Grey line: SGH10-p. Pink line: SGH10. Yellow line: Media only control.

3.2.9 In vivo assessment of virulence using the zebrafish model

3.2.9.1 *Survival after infection by immersion*

Next, we used zebrafish larvae infection by immersion to assess the virulence *in vivo* of the three strains designated for this study, as it has been shown as a reliable model in order to study the dissemination and lethality caused by bacterial strains.

We first observed the survival of these larvae in response to infection with our strains. As shown in **Figure 3.11**, we can see that survival of zebrafish larvae immersed into SGH10 was significantly less than the those immersed into SGH10-p and Ecl8, confirming the lower virulence of those two strains.

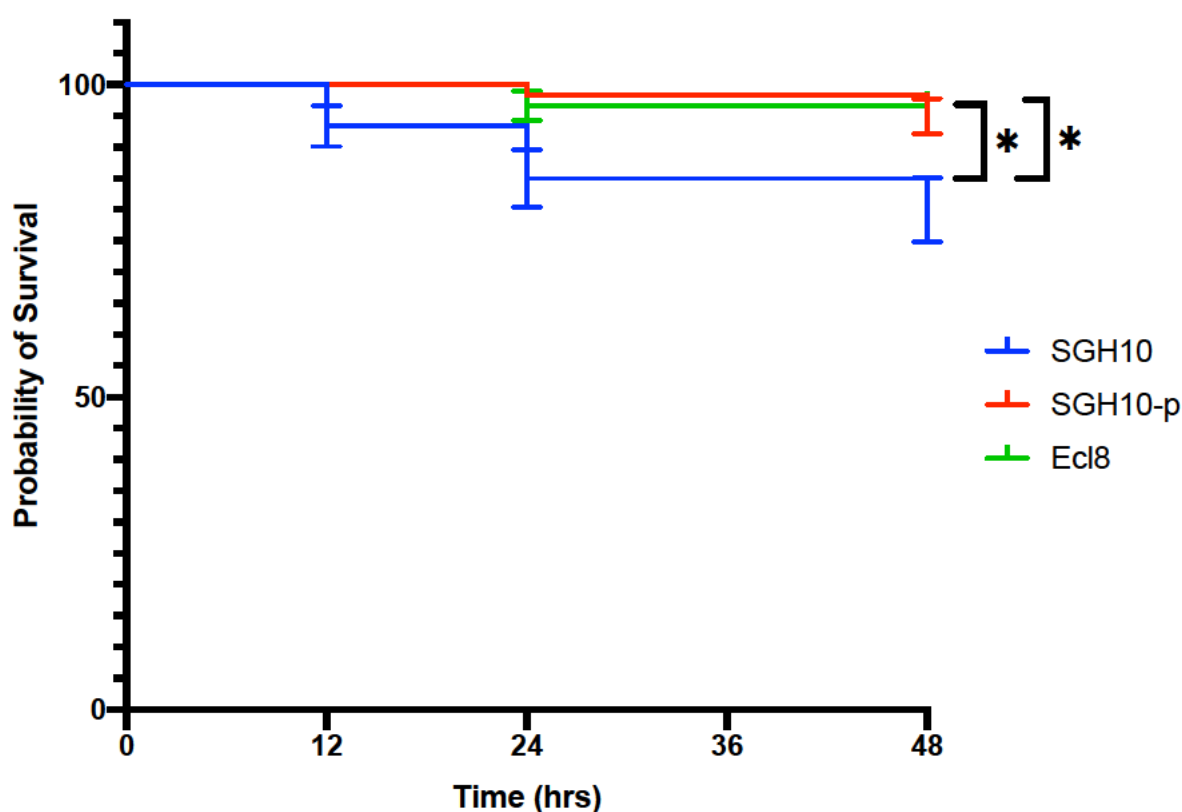


Figure 3.11. Survival curve of zebrafish larvae

After infection by immersion (10^9 cfu/ml) of zebrafish larvae ($n=20$) per replicate with SGH10, SGH10-p and Ecl8. Bars represent standard error of three biological replicates, compared with Log-rank (Mantel-Cox) chi-square test. *adjusted $p < 0.05$.

3.2.9.2 Bacterial dissemination after infection by immersion

We then measured dissemination of our 3 strains in zebrafish larvae after 24 hours of infection by immersion, as this provided more information about host-pathogen relationships than a simple survival test. After 24-hour infection, the 20 infected embryos were vigorously washed to minimise the number of bacteria adhered to the outside of the larvae. We observed a wide variability between biological replicates, as shown by large error bars on **Figure 3.12**. Although not significant, this assay nevertheless showed a correlation with the data on larval survival, with significantly greater dissemination for SGH10 compared with Ecl8.

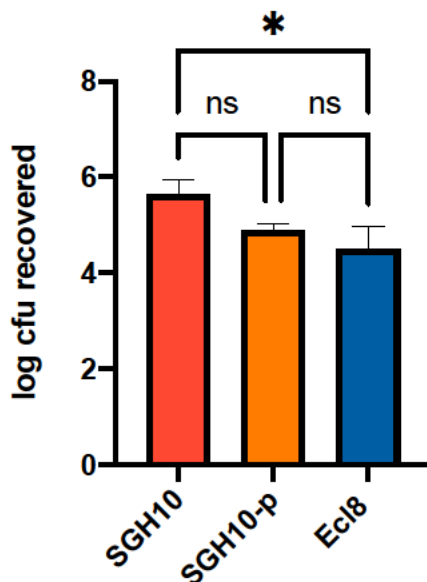


Figure 3.12. Log of CFU counted after infection of zebrafish embryos.

After infection by immersion (10^9 cfu/ml) of 20 zebrafish larvae per replicate with SGH10, SGH10-p and Ecl8. Bars represent standard error of three biological replicates, compared with one-way ANOVA with multiple comparison. *adjusted $p < 0.05$.

3.3 Discussion

The objective of this chapter was to characterise and validate the three strains to develop our ECIS model, examining their bacteriological properties and also host-pathogen interactions. Overall, we were able to confirm that (1) SGH10 displayed all the characteristics of *HvKp*, (2) that the loss of the virulence plasmid altered these traits, despite the unexpected conservation of a thick capsule and that (3) Ecl8, although retaining and expressing hypervirulence genes (including *mpA*, *mpC* and *iuc*), was neither HMV or hypercapsulated and did not display any of the phenotypic or pathogenicity characteristics of SGH10.

First of all, the capsulation and viscosity levels of our strains were unexpected, but ultimately useful for the development of our model (see Chapter 4). While the high level of capsulation and HMV in SGH10 was expected, we assumed that the loss of the virulence plasmid (and therefore of the *mpACD* locus) would result in the loss of both the hypercapsule and the HMV.

Unpredictably, SGH10-p remained hypercapsulated, with the expression of the *cps* locus staying high. This means that whatever is necessary for hypercapsulation is not on the virulence plasmid, but probably due to genetic characteristics on the chromosome of SGH10. This could involve genes either precisely in the *cps* locus or other regulatory ones (e.g. *kvrAB* or *rscAB*), allowing the hypercapsule to be retained without the positive regulation of *mpA* and *mpC*.

Ecl8 was neither hypercapsulated nor HMV, as previously demonstrated.²⁵⁵ Contrary to what was thought, this was not due to the expected frameshift mutation in the *mpACD* locus as described in the section 3.1, as both *mpA* and *mpC* are expressed.

The only factor we could identify explaining why Ecl8 lost its a thick capsule was the significant reduce in the expression of *wzi*, coding for a protein anchoring the capsule to the cell surface. However, we do not know whether this is linked to a defect in *wzi* specific to Ecl8 or whether it is the result of the loss of a potential positive regulator of *wzi* (no precise ones being known in the literature to date).

Overall, many factors could explain these genotype-phenotype discrepancies, as both the hypercapsule and HMV have been shown to depend not only on the *cps* locus and classical regulators, but also on many other metabolic genes, including genes involved in Krebs' cycle, intracellular transport, protein and lipid biology.¹⁵²

In terms of the growth of our strains, the three strains showed relatively similar growth in the cell culture medium, which do not contain the same ingredients as conventional bacterial growth media (for instance containing glutamate or DMSO). The slight reduction in growth observed with SGH10 and SGH10-p can probably be explained by the high metabolic cost of their thick capsule.^{118,152,257,258} While these differences had no impact in the adhesion and internalisation assays (because bacteria were resuspended in PBS), they had to be kept in mind in the subsequent analysis of the more advanced infection assays described in chapters 4 and 5.

These “simple” host-pathogen adhesion and internalisation tests were informative as they reflected that the high levels of both capsule and hypermucoviscosity impede adhesion.^{147,259} Interestingly, HMV has recently been distinguished from hypercapsule, and appears to be the main factor determining the avoidance of host cells association.^{152,245,260} This was reflected in our results, showing a clear difference in adherence between SGH10 and SGH10-p, only differing in terms of HMV. Yet, the

capsule also played a role in hindering adhesion to host cells, as Ecl8 was still adhering significantly less than SGH10-p.

Overall, it was interesting to distinguish two patterns of relationships between bacteria and epithelial cells, depending on their structure and function. Intestinal and endothelial cells, which have more of a barrier function, showed consistent adhesion differences between the 3 strains, with a clear role for both the capsule and HMV; whereas the adhesion of the strains to HepaRG liver and biliary ducts cells did not involve the capsule, as illustrated by the absence of difference between SGH10-p and Ecl8 (**Figure 3.7**). This could be explained by the fact that, although hepatocytes can be considered as epithelial cells, they have less of a barrier and more of a metabolic function, therefore presenting different interactions with bacteria.

In addition, bacteria were internalised by intestinal cells, but not in liver cells. This internalisation was severely hindered both by the capsule and HMV, irrespective of the level of adhesion.

Altogether, the data collected from the adhesion and internalisation assays suggested that the pathophysiological processes with gut or liver derived cells are distinct, with specific roles of both the HMV and capsule.

Another main virulence feature of *HvKp* is its avoidance of the immune system. To study this feature in the three strains, we carried out a chemotaxis test using macrophages. Despite the limitation of a one-off experiment, we found Ecl8 attracted MDM much more than SGH10 and SGH10-p, and that the loss of the SGH10 virulence plasmid did not affect its ability to avoid the immune response. This could easily be

explained by the fact that SGH10-p retained a thick capsule, known to be the critical factor in avoiding phagocytosis.

Finally, we completed the characterisation of our strains by using them to infect zebrafish larvae *in vivo*. Both the survival curves and the dissemination in the larva after infection by immersion showed staggered levels of virulence between SGH10, SGH10-p and Ecl8. The main limitation of these results was their high technical variability, particularly in the study of bacterial dissemination. This is probably due to the very nature of the immersion infection model, with many variables over which we have no control (quantity of bacteria ingested, larval motility, etc). Yet, the observed differences were probably effects of the capsule and HMV.

These comparisons also required siderophores to be taken into account, as the siderophore aerobactin has been shown to influence the virulence (both in terms of lethality and dissemination) of *HvKp* in animal models.^{111,112} We could however see that Ecl8, although still secreting aerobactin, showed only a low level of virulence, similar to that of SGH10-p, which did not secrete aerobactin but retained a level of capsulation. This illustrates that capsulation and HMV are probably more important factors than aerobactin in the interactions with gut or liver cells.

In summary, this chapter has enabled us to define and justify the use of SGH10, SGH10 without virulence plasmid and Ecl8 as relevant strains to establish a *HvKp* infection model. These strains vary in terms of capsulation and hypermucoviscosity, which we were able to confirm as determining factors in the interactions with epithelial and immune cells *in vitro*, as well as in virulence in an *in vivo* model.

Chapter 4: Implementation of an impedance assay-based model for studying host-pathogens interactions between gut/liver cells and HvKp

4.1 Introduction

Host-pathogen interactions refer to the complex biological and molecular balance between a host organism and a pathogen. These interactions will determine the outcome after a pathogen enters the host, which can be either its elimination or the causation of an infection.^{261,262} The human body has many lines of defence to prevent an organism from initiating an infection. One of these is the epithelial barrier, a tight cellular barrier which prevents and regulates the passage of solutes or pathogens from the outside environment to internal body systems.^{263–265}

HvKp has been shown to colonise the intestine before forming an abscess in the liver, reached via the portal circulation.^{197–199} Its pathophysiological process therefore requires crossing this intestinal barrier, whose major defence factors are precisely the gut and vascular epithelia. Thus far, the understanding of the interactions between the gut epithelia and HvKp are limited. Similarly, the way in which HvKp affects liver cells and forms abscesses is poorly defined.

Although animal models are still the most reliable approach for studying these host-pathogen relationships, they are time-consuming, expensive, and the biological relevance to humans is not always clearly established.^{266,267} A number of *in vitro* techniques using human-derived cell lines have thus been developed, providing greater insights into the host-pathogen interactions. In ascending order of complexity,

these consist of planar monoculture, co-culture, three-dimensional culture, organoids and organs on-a-chip.²⁶⁸ The choice of the technique to use depends on the subtle balance between a system that is simple and sensitive enough to reveal pathogen mechanisms but has sufficient complexity to be relevant *in vivo*.^{269,270}

The tools and techniques to observe and explore these *in vitro* models are diverse, each offering insights into cellular behaviour, molecular interactions, and the effects of various substances or pathogens on biological systems. They are ranging between (1) microscopy, to visually assess these interactions and their consequences on eukaryotic cells, (2) Western blot, mass spectrometry, ELISA or flow cytometry to detect and analyse proteins, hormones, antibodies, antigens or other cell secreted or immune system elements, indirectly providing information on the infection process and (3) assessment of the integrity of eukaryotic cells (i.e. viability, permeability, proliferation, etc.) using cell culture (probe) assays or electrophysiological techniques.

In that context, impedance-based assays have long been used for the study of host-pathogen interactions.^{271–276} By measuring the electrical impedance generated by cell layers, changes in cell health, morphology, and permeability can be detected, offering insights into the mechanisms of infection and the host's response to pathogenic challenge.^{271–276}

One such impedance-assay is ECIS, described in detail in section 1.7.1. Briefly, eukaryotic cells generate resistance against a current applied at various frequencies. Resistance at low frequency current reflects the adhesion of the cells to the substrate and the integrity of tight junctions, while resistance at higher frequencies indicates the integrity of the cell membrane. Primarily, ECIS has been used in various fields of biomedical research, including wound healing studies, toxicology, cancer research,

and the exploration of cellular responses to pharmacological agents.^{271,277,278} Its ability to provide continuous, quantitative data on cell behaviour under physiological conditions makes it a precious tool for understanding the complex dynamics of cell function. Whilst ECIS has already been used to investigate host-pathogen interactions, it has never been experimented with *HvKp*.

Bacterial specific toxic effects on intestinal cells had already been demonstrated for other gut pathogens, using various techniques. For instance, *Salmonella typhimurium* has been shown to redistribute TJ proteins to disrupt the intestinal epithelial barrier and cross through a paracellular route, which is unique and clearly contrasts with the absence of damage caused by commensal bacteria to the epithelium.^{279,280}

HvKp's gut colonisation and crossing are probably the most crucial stages in its pathophysiology. Our main hypothesis was therefore that *HvKp* has specific interactions with gut (Caco-2) and liver (HepaRG) cells, and the first aim of this chapter was to develop a model based on ECIS to study how it specifically interacts with the gut. The second objective of this chapter was to use this model to investigate the damage of *HvKp* on liver cells leading to abscess formation.

4.2 Results

4.2.1 Monitoring of host-pathogen interactions between gut, liver and endothelial cells and HvKp using the impedance-based ECIS assay

4.2.1.1 *Direct infection on ECIS*

Using the strains SGH10, SGH10-p and Ecl8, which differ in capsulation & HMV levels (see **Table 3.2**), we set out experiments to optimise the ECIS setup.

For an initial overview and to fine-tune the technical settings, we directly infected intestinal, liver or endothelial cells (Caco-2, HepaRG and HUVECS cells, respectively) in ECIS 8-well plates. We then monitored the resistance generated to different current frequencies (4kHz, reflecting TJ integrity, and 64kHz, reflecting cell membrane integrity) over time. An example of the curve obtained is illustrated in **Figure 4.1**, displaying the resistance generated by the cell layer (HepaRG in this case) decreasing over time under the effect of the bacterial infection. Visually, we could already see that the decrease in resistance caused by the infection of our liver cells by SGH10 was steeper and faster than that of the other two strains. Yet, a proper method to quantitatively analyse the data was necessary.

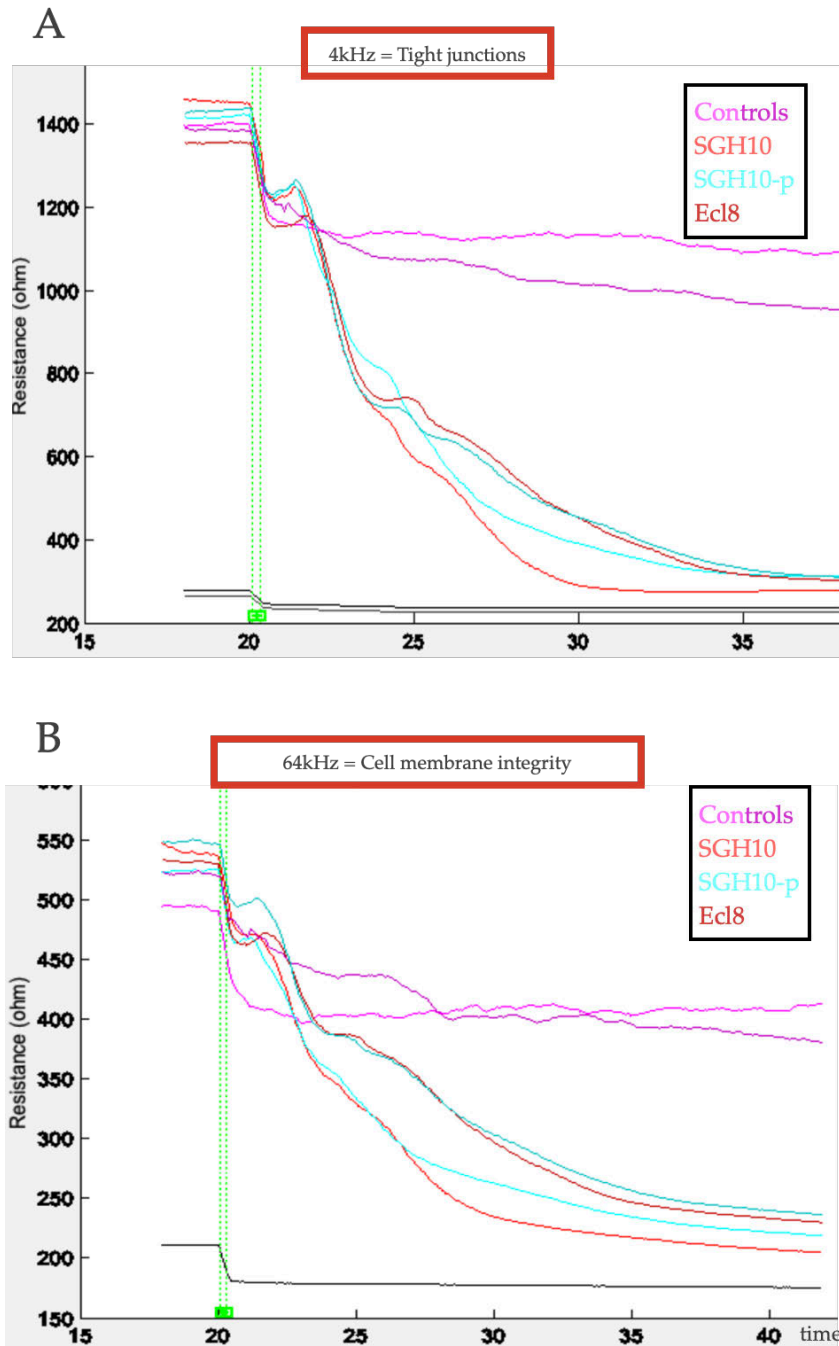


Figure 4.1. Examples of ECIS curves obtained in the ECIS software after infection of HepaRG with SGH10, SGH10-p and Ecl8.

A. Resistance over time in hours recorded at 4kHz, reflecting mostly tight junctions' integrity.

B. Resistance over time in hours recorded at 64kHz, reflecting mostly cell membrane integrity.

Black line: No cells control. Light and dark pink lines: Uninfected HepaRG control. Red & dark blue lines: HepaRG infected with SGH10. Turquoise line: HepaRG infected with SGH10-p. Burgundy line: HepaRG infected with Ecl8.

4.2.1.2 Normalisation of the data and determination of T50 decrease factor

ECIS is a particularly interesting technique because it allows the dissection of cell-pathogen interactions by measuring the variations in cellular electrical resistance in the presence of the pathogen over time and to specifically look at TJ or cell membrane integrity by using different voltage. However, if a trend is often “visually” observed over time (see **Figure 4.1**), no precise method to quantify it exists. Some authors limit themselves to this visual qualitative analysis, observing trends but not quantifying effects.^{210,218,247}

Others use the area under the resistance curve or the average of all resistance values over a certain period of time.²¹³ Unfortunately, all these methods fail to take into account several technical variations and do not reflect the biological phenomenon. For instance, the change of cell media and the addition of bacteria cause a net increase in resistance, with inconsistent amplitude which is not associated with any biological effect. Similarly, despite normalisation, the resistance values obtained both at the plateau phase of eukaryotic cells and when these are damaged vary between wells.

We first tried to measure and plot the area under the resistance curve (AUC) (**A**) or a coefficient consisting of the division of the timepoint when the resistance starts decreasing by the angular coefficient of the slope (**B**). For these two methods, we expected an inversely proportional relationship between the harmfulness of bacteria on eukaryotic cells and the AUC or the coefficient.

Figure 4.2 illustrates the results obtained with Caco-2 cells at 4kHz, and shows that (1) the AUC does not work, as even a difference with the control (uninfected cells) cannot be demonstrated, and that (2) the coefficient seems to show differences

between the effects of the three bacteria, but presents with a considerable variation, making this difference non-statistically significant. This high degree of variability was to be expected, since a small variation in either the starting point for the reduction in resistance or the slope will significantly alter the value of this coefficient.

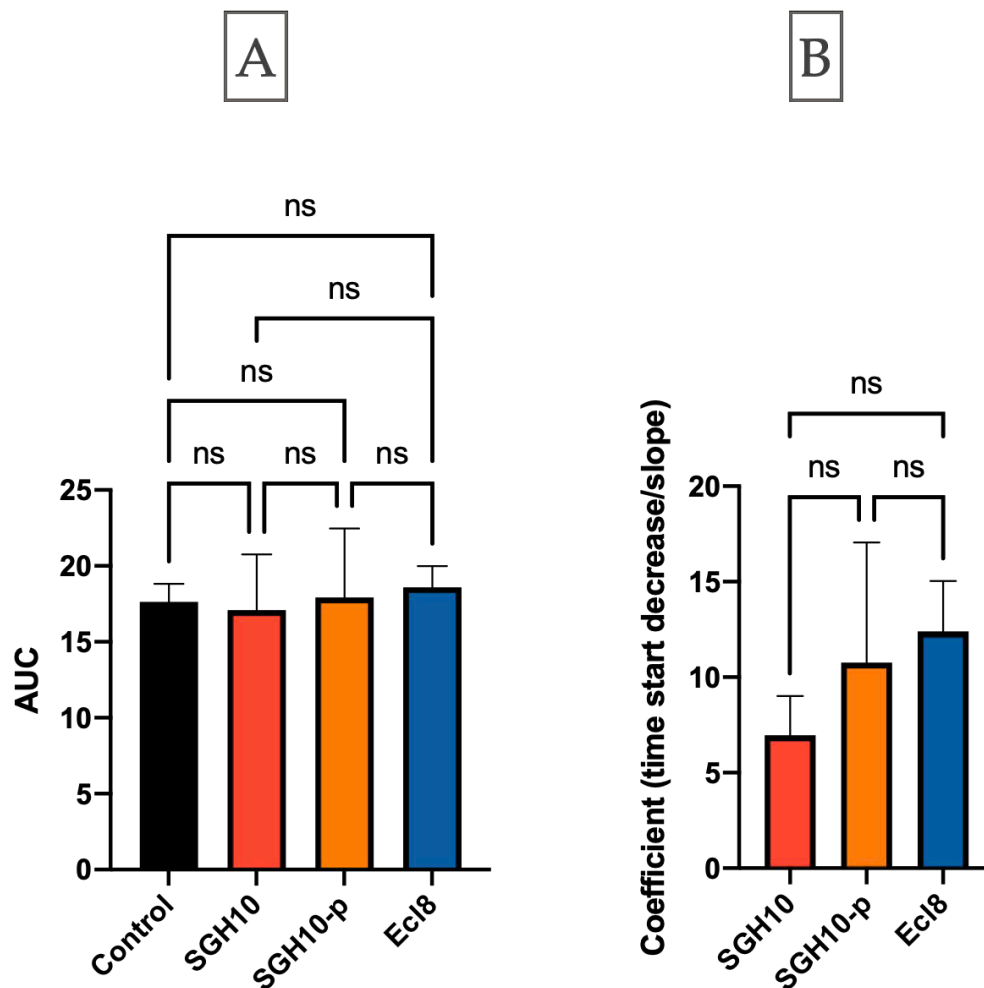


Figure 4.2. Effect of bacteria on tight junctions (4kHz) of Caco-2, measured by the area under the curve (A) or a coefficient (time when decrease of resistance starts/slope of the decrease).

Bars represent mean and standard deviation of two technical and three biological replicates, compared with a one-way ANOVA test with multiple comparisons. ns = non-significant.

As these calculation methods couldn't prevent these artefacts from concealing the true biological effects, we developed what we called the "T₅₀ decrease factor" (T₅₀DF), defined as the time necessary for a reduction of 50% of the amplitude of the normalised resistance curve over time. The amplitude was determined as the difference between the highest and the lowest value of the curve. Normalisation consisted of designating the time point at which bacteria were added as 0, and the corresponding resistance measured at the plateau phase as 1 (detailed in chapter 2). A graphical representation of this T₅₀DF determination can be found in **Figure 4.3**, representing resistance curves obtained after normalisation of the data and gathering of three biological replicates. It illustrates that the main advantage of this method is that it considers both the time at which the effect starts, but also how intense it is (i.e. how steep is the decrease of the curve), irrespective of the unpredictable artefactual blips.

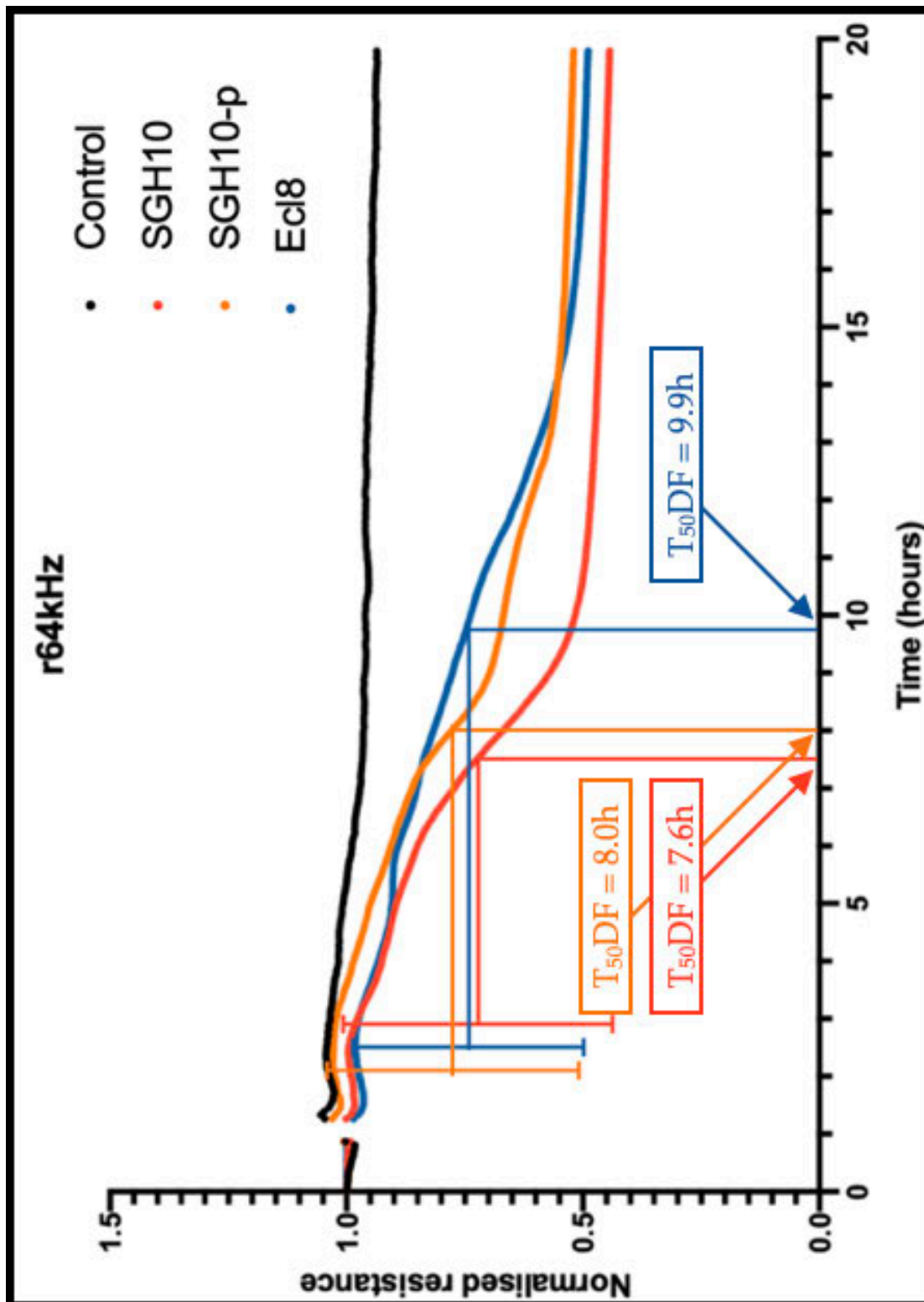


Figure 4.3. Graphic representation of how the T_{50DF} is determined.

Three biological replicates were pooled (error bars not shown for readability). First, the amplitude of the normalised resistance was determined, then the T_{50DF} (time corresponding to half of this amplitude) was extrapolated.

4.2.1.3 *HvKp specifically disrupt gut cells TJ*

Having rigorously established the methods for analysing the results obtained with ECIS, we were able to move on to the actual experimental setup. Considering first the intestinal cells (Caco-2), addition of EGTA (ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, a calcium chelator disrupting TJ) allowed us to confirm that the 4kHz frequency did indeed reflect the changes in integrity of the TJs, since this resulted in a significantly lower T_{50DF} than bacteria, which was not observed at 64kHz.

When comparing our three strains, we observed that SGH10 had a significantly greater effect at 4kHz, meaning that it was inducing TJ disruption at least 2-3hrs earlier relative to SGH10-p and Ecl8 (**Figure 4.4**). No difference in T_{50DF} was noticed between the highly capsulated SGH10-p and the normally capsulated Ecl8, suggesting that HMV is the principal characteristic causing this effect on cell integrity. When looking at the resistance at high frequency (64kHz), we could see there was strictly no difference between strains in terms of cell membrane toxicity. (**Figure 4.4**)

4.2.1.4 *The thick capsule potentiates damages to hepatocytes*

As shown in **Figure 4.4 D**, both SGH10 and SGH10-p impacted TJ and cell membrane integrity in HepaRG cells significantly earlier than Ecl8. However, comparing the effects of SGH10 and SGH10-p showed that, while the disruption of TJ was higher for SGH10 relative to SGH10-p (similar to the effect observed on Caco2's), the damage to cell membrane was not significantly different between them. This potentially indicated that capsulation, more than HMV, was responsible for this difference. Because HepaRG are comprised of both hepatocytes and epithelial cells

(cholangiocytes), we tried to untangle the contribution of each of these cell types by performing ECIS using HepG2/C3A, a cell-line containing only hepatocytes. Unlike HepaRG, no differential effect between SGH10 and SGH10-p was noticed at 4kHz (**Figure 4.4G**). This suggested that the effect of SGH10 and SGH10-p observed on HepaRG cells TJ was still predominantly due to the epithelial cell component of HepaRG (cholangiocytes), and didn't reflect the effect of HvKp on hepatocytes.

This was also supported by analysis of EGTA T₅₀DF, which showed significant toxicity to HepaRG at 4kHz, similar to that of SGH10.

Cell membrane integrity, on the other hand, was also decreased in HepG2/C3A cells after infection with SGH10 and SGH10-p compared to Ecl8. This was then probably linked to an effect on the hepatocytes. Again, the lack of difference between SGH10 and SGH10-p suggested that the capsule was a key element in the generation of this increased overall toxicity of HvKp on liver cells.

4.2.1.5 HvKp has no specific effect on HUVECS cells or bronchial epithelial cells.

The final element we wanted to elucidate using ECIS was whether the effects of HvKp on epithelial cells were specific to the gut-liver axis or if they could be applied to any epithelial cell type. We first looked at the differences in virulence of our strains on HUVEC endothelial cells. This is important because the endothelial barrier must also be crossed by HvKp as it enters the liver. Interestingly, this showed no difference between our strains, neither for cell membrane integrity or, more importantly, tight junction function (see **Figures 4.4E and F**). Similar findings were observed after infection of epithelial cells completely independent of the intestine-liver axis, in this case 16HBE, a bronchial epithelial cell line (**Figure 4.4I and J**). Overall, these results

interestingly indicated that the differential effect of *HvKp* was limited to gut and liver epithelial cells.

*4.2.1.6 These results were reproducible with another *HvKp* strain*

Different strains of *HvKp* can vary relatively widely from one another, since most of their genome contains accessory genes. We therefore wanted to countercheck the $T_{50}DF$ obtained with SGH10 using another hypercapsulated and hypermucoviscous strain, KPPR1. KPPR1 is widely studied for understanding bacterial pathogenesis due to its ability to cause pneumonia and bacteriemia but also thanks to the fact that it can easily be genetically modified.^{281,282} As shown in Figure 4.5, we obtained similar results between these two *HvKp* strains.

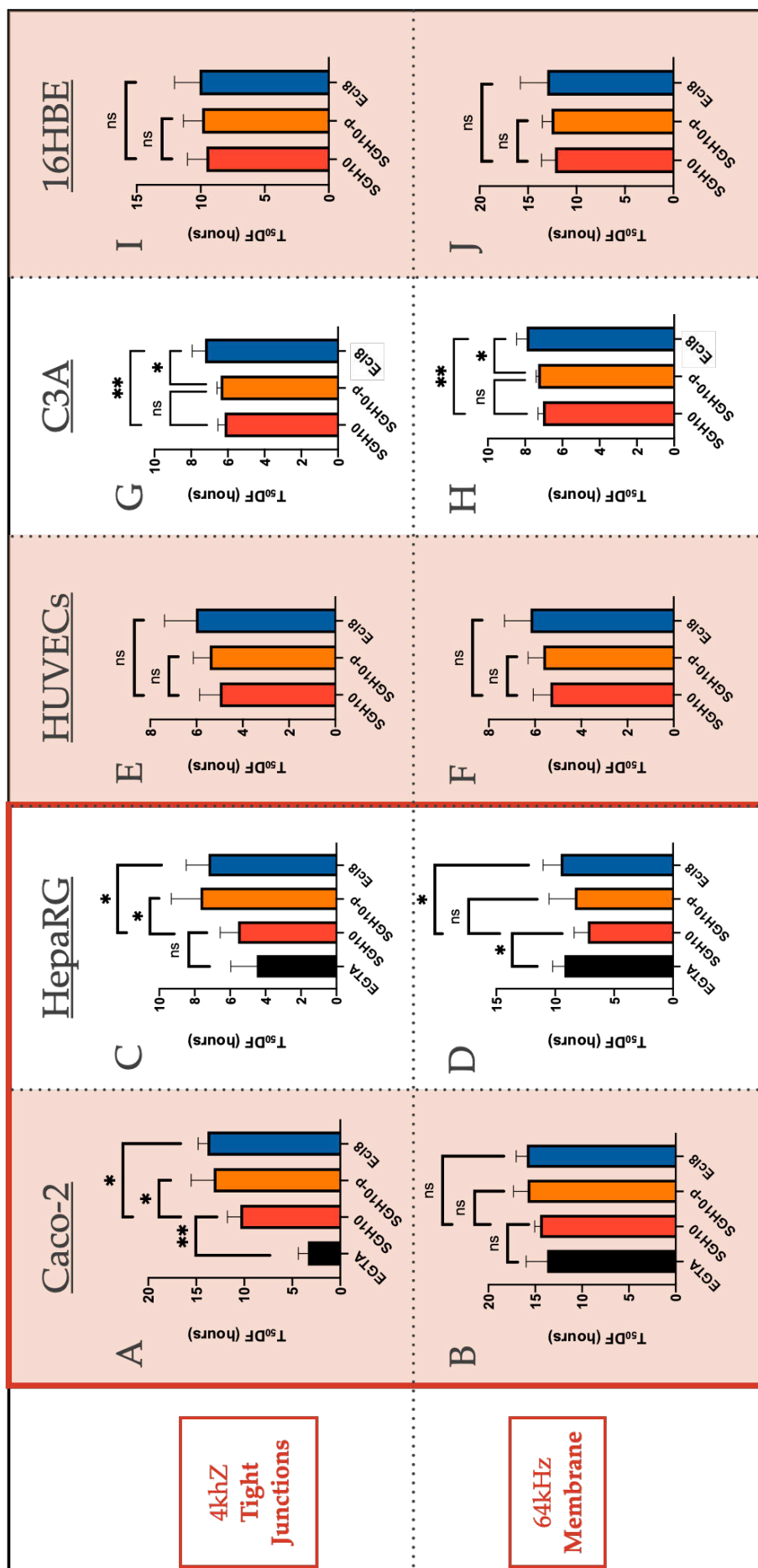


Figure 4.4. Effect of bacteria on tight junctions and cell membrane integrity of Caco-2, HepaRG and HUVECS cells, recorded by ECIS.

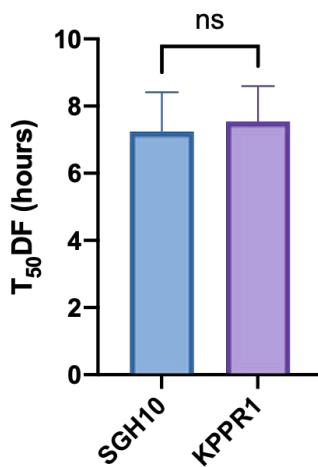
Epithelial cells highlighted in pink.

Current frequency of 4kHz (TJ changes): A. Caco-2 cells. C. HepaRG cells. E. HUVEC cells. G. C3A cells. I. 16HBE cells.

Current frequency of 64kHz (Membrane integrity changes): B. Caco-2 cells. D. HepaRG cells. F. HUVEC cells. H. C3A cells. J. 16HBE cells.

Bars represent mean and standard deviation of two technical and five biological replicates, compared with a one-way ANOVA test with multiple comparisons. ns = non-significant. *adjusted $p < 0.05$. Non-significant statistical tests not reproduced on the figure.

HepaRG - 64kHz



Caco-2 - 4kHz

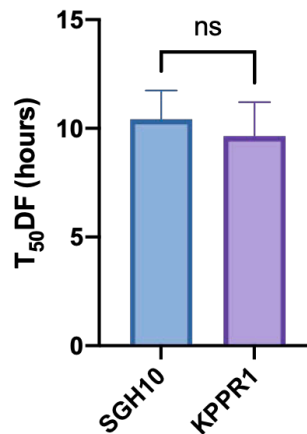


Figure 4.5. Comparison of T₅₀DF of SGH10 and KPPR1 on HepaRG and Caco-2. At frequency of 64kHz on HepaRG (left panel) or frequency of 4kHz on Caco-2 (right panel). Five biological replicates.

4.2.2 Confirmation of the gut TJ greater disruption by HvKp

Using ECIS, we revealed a specifically greater disruption of TJ caused by HvKp, with indications that this is might be linked to its HMV phenotype (see section 4.2.1.3). To confidently develop and further use our system, this required confirmation using alternative techniques such as immunohistochemistry and TEER (TransEpithelial Electrical Resistance), a well-known and described technique for assessing the integrity of tight junctions.

4.2.2.1 *Using ICC*

We first tried to use an immunohistochemistry method, probing TJ's ZO-1 proteins from infected intestinal cells with primary antibodies, detected by fluorescent secondary antibodies, and subsequently visualising and quantifying them. While there was a general visual impression that the TJs were more degraded after infection with SGH10

(see **Figure 4.6**), multiple attempts at computer quantification of this fluorescence were unsuccessful. This was mainly due to the low quality and intensity of the staining and the many artefacts present in the field. To improve this, we tried various modifications to the protocol such as using another ZO-1 primary antibody, using plates with larger wells, changing the growth time of the Caco-2 cells (7, 14 or 21 days), changing the infection duration, and even changing the microscope (using a confocal). This was however unsuccessful, and **Figure 4.6** displays the best image we could obtain on EVOS, infecting our cells for 3 hours in a 12-wells plate, using the ThermoFisher 61-7300 anti-ZO-1 antibody and following the method described in section 2.3.4.2.

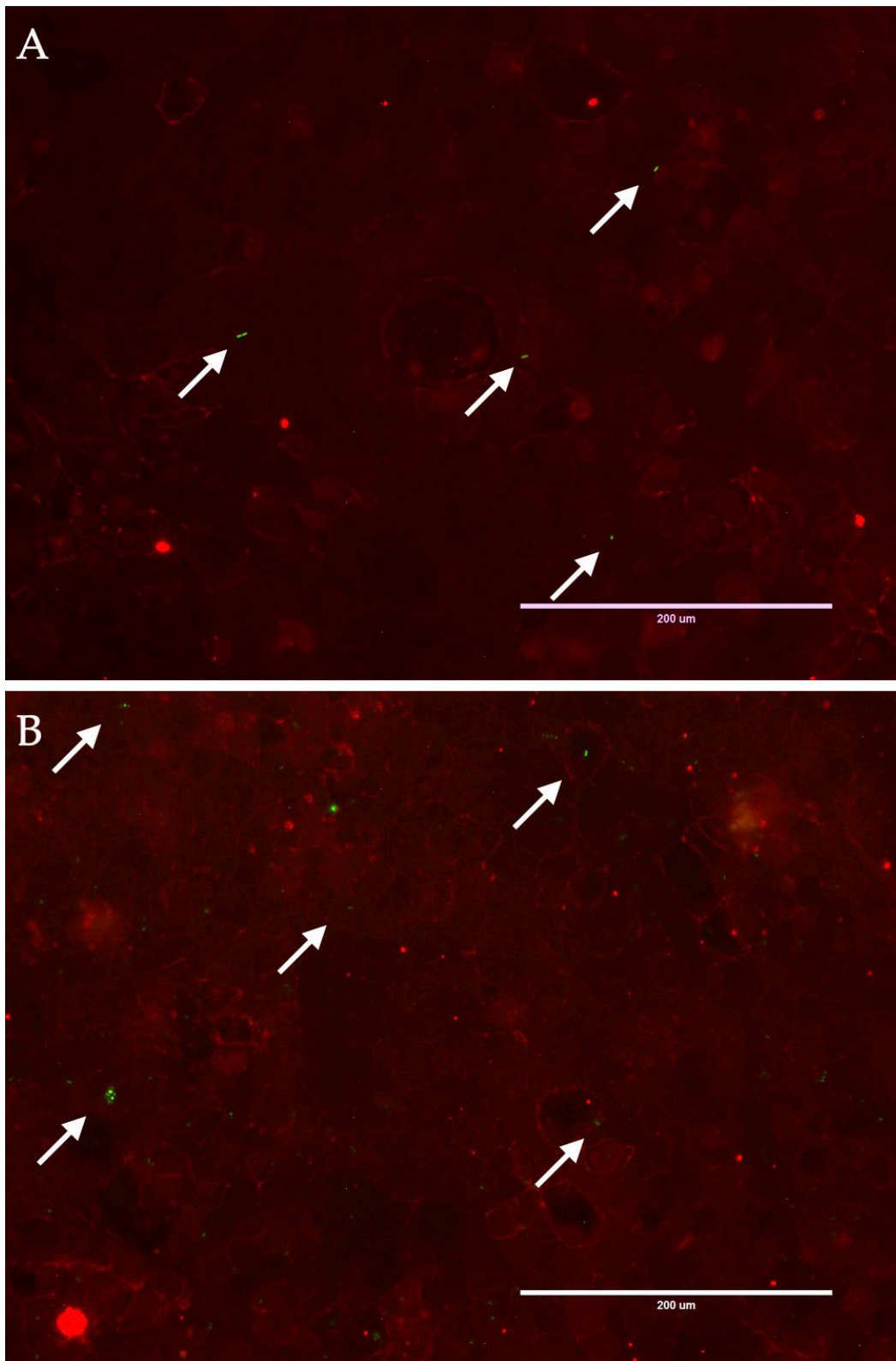


Figure 4.6. Caco2's cells ZO-1 staining after 3-hours infection by SGH10 or SGH10-p, captured on EVOS. Visually suggesting weaker and less continuous staining for SGH10 (Panel A) compared to SGH10-p (Panel B). White arrows are indicating GFP-tagged bacteria.

4.2.2.2 Using TEER

Given the lack of success of the ICC method, we tried to confirm the greater gut TJ's disruption caused by *HvKp* by using a different method called TEER. This consists of recording the resistance between two chambers, generated by a cell layer grown on a Transwell insert separating them (see **Figure 2.5.** in section 2.3.7). Interestingly, the main determinant of this resistance has been shown as the TJ integrity, with other factors (such as cell membrane) contributing significantly to this resistance.^{209,283–286} Caco-2 cells were seeded into the insert, and the TEER generated was checked every 2 days until a plateau phase was observed (constant value of resistance over time, generally after 10 days). The Caco-2 cells present in the insert were then infected, and the TEER recorded for 5 hours post-infection.

The results, shown in **Figure 4.7**, confirmed the ECIS data by demonstrating that infection of Caco-2 cells by SGH10 resulted in a 2-fold decrease in resistance after 4 hours, which was comparable to EGTA positive control, a known tight junction disrupter, alone. This decrease in resistance was also significantly greater compared to SGH10-p and Ecl8, which were not significantly different from non-infected cells (**Figure 4.7**).

These results therefore made the observations using ECIS more robust, asserting that *HvKp* disrupted TJs in intestinal cells more than both its version lacking virulence plasmid and *cKp*. Interestingly, as for the ECIS results, TEER confirmed the difference between the effect of SGH10 and SGH10-p. Given that the loss of plasmid between SGH10 and SGH10-p removes the HMV phenotype, the effects recorded by both ECIS

and TEER measurements confirmed the crucial role of this phenotype in bacteria-epithelial cells interactions.

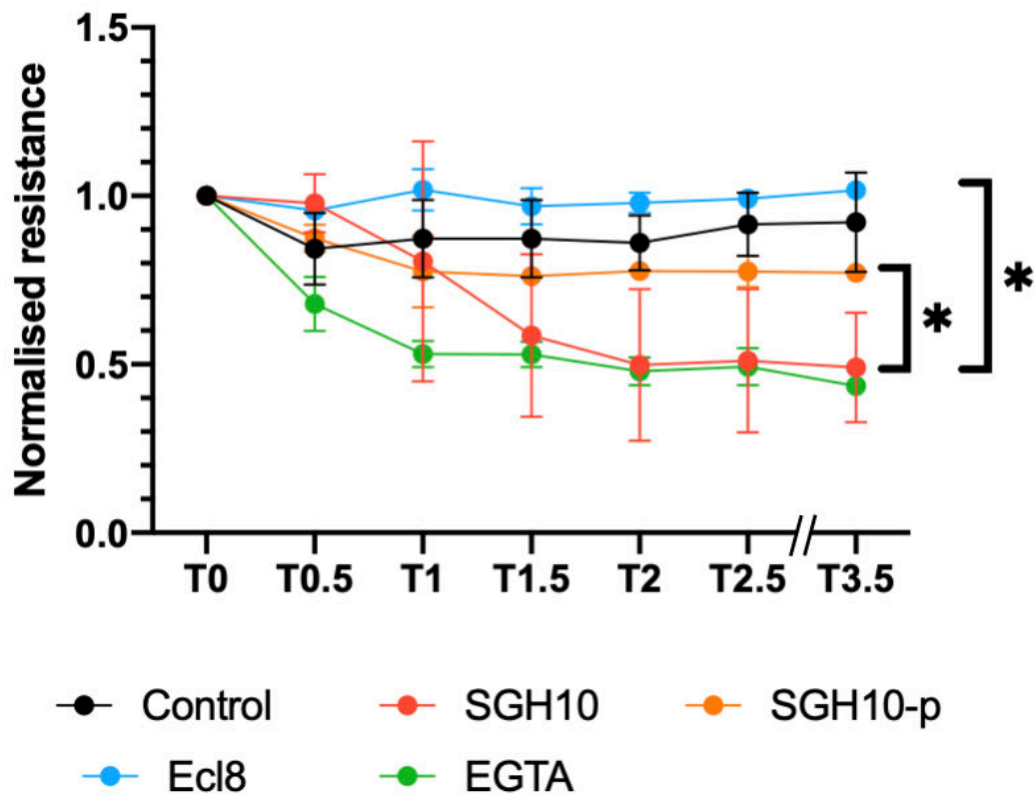


Figure 4.7. Evolution of the normalised transepithelial resistance generated by Caco2's over time, after infection.

Black line: non-infected well control. Red line: Caco-2 infected with SGH10. Orange line: Caco-2 infected with SGH10-p. Blue line: Caco-2 infected with Ecl8. Green line: EGTA. Bars represent mean and standard deviation of two technical and three biological replicates. Statistical analyses performed using mixed effects models to avoid auto-correlation
 *adjusted $p < 0.05$. Non-significant statistical tests not reproduced on the figure.

4.2.3 Confirmation of liver cell toxicity

Using ECIS, we previously shown that SGH10 and SGH10-p are specifically damaging the hepatocytes' membrane (section 4.2.1.4). We aimed to countercheck this biological effect with another method that would also allow us to focus on this membrane integrity, overlooking the state of the TJs.

4.2.3.1 PrestoBlue, LDH secretion and CellTox cytotoxicity assays

Confirmatory experiments were undertaken using simple eukaryotic cells viability assays, i.e. (1) The PrestobluTM assay, which is a non-lytic assay, measuring cell proliferation, viability and metabolic activity through quantifying the reduction of the blue, non-fluorescent, resazurin into the red-fluorescent resorufin by mitochondrial cellular respiration (NADPH, FAD, NADH and FMNH).²⁸⁷ (2) The lactate dehydrogenase (LDH)-GloTM Cytotoxicity Assay, a bioluminescent method that measures LDH levels released from cells into culture media, indicating plasma membrane disruption and cell damage. (3) The CellToxTM Green Cytotoxicity Assay, which measures cell death by detecting changes in membrane integrity by employing a unique cyanine dye that enhances fluorescence when binding to DNA in compromised cells, with the intensity of this fluorescence directly correlating to the level of cytotoxicity. Alas, **Figure 4.8** shows that none of these 3 tests demonstrated any statistically significant difference in terms of HepaRG viability between the 3 strains, despite their difference in virulence, extensive troubleshooting and adaptation of protocol. As further expanded in the discussion (section 5.3), this is probably down to interference from the high number of bacteria present in the mix.

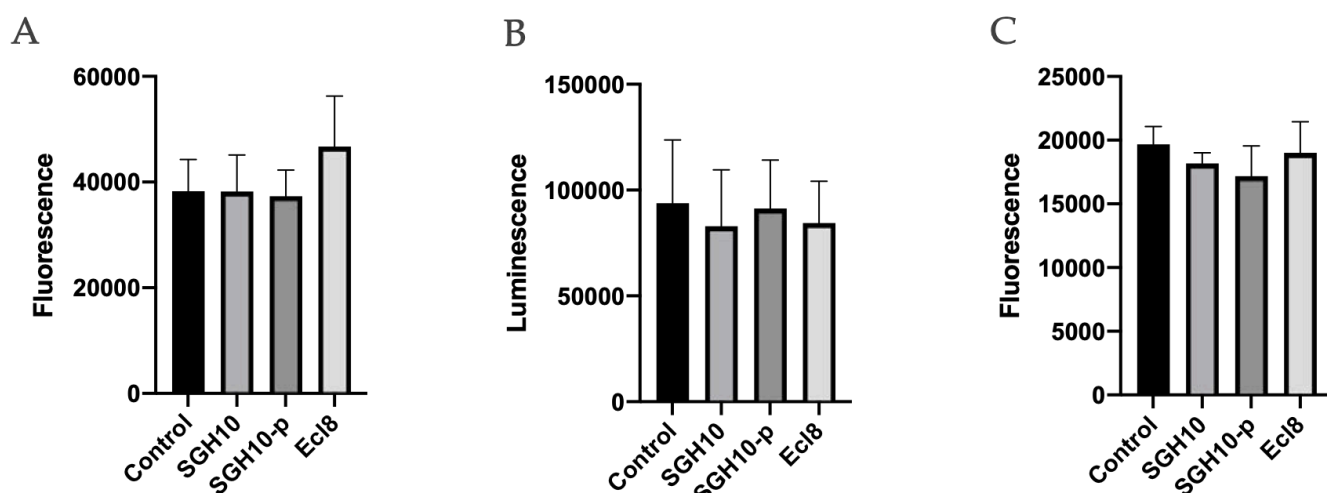


Figure 4.8. Results of the Prestoblu™ assay (A), LDH-Glo™ Cytotoxicity Assay (B) and CellTox™ Green Cytotoxicity Assay (C) after infection of HepaRG with SGH10, SGH10-p and Ecl8.

Control = Uninfected cells. Three technical and biological replicates. Statistical analyses not shown on graph as no significance was found.

4.2.3.2 Flow cytometry

We hypothesised that the reason why the viability tests carried out previously were not highlighting any difference between the three strains was that they were unable to distinguish between eukaryotic cells and bacteria. We thus designed an experiment using flow cytometry, precisely because this technique appeared more suitable for discriminating between these cells and SGH10, SGH10-p and Ecl8.

After infection, the cells were lifted from the plate and first stained with the HLA (Human Leukocyte Antigen) targeting antibody HLA_IPE_Cy7, to identify eukaryotic cells and exclude the substantial bacterial interference. We then used SybrGreen to gauge the viability of these eukaryotic cells.

An example of the results and the gating we performed is shown in **Figure 4.9**. We then compared the percentage of live HepaRG after infection with our strains. As some dead cells can be turned into debris and therefore no longer detected by the machine, we first ensured that the number of cells on which the life/death distinction was made was comparable between the different conditions. This is shown in **Figure 4.10A**, confirming that the number of cells analysed was similar for all three strains, significantly higher than the positive control Triton X-100.

Once this was established, we were able to confirm the higher toxicity of SGH10 on HepaRG compared to other strains, as its infection was resulting in a significantly lower percentage of live cells compared to the two other strains (65% vs 80%, see **Figure 4.10B**). However, unlike ECIS, this experiment showed that the damage caused by SGH10 and SGH10-p was significantly different. As further detailed in the discussion, this contradiction between ECIS and flow cytometry probably demonstrates the role of both the capsule and HMV in liver cell toxicity.

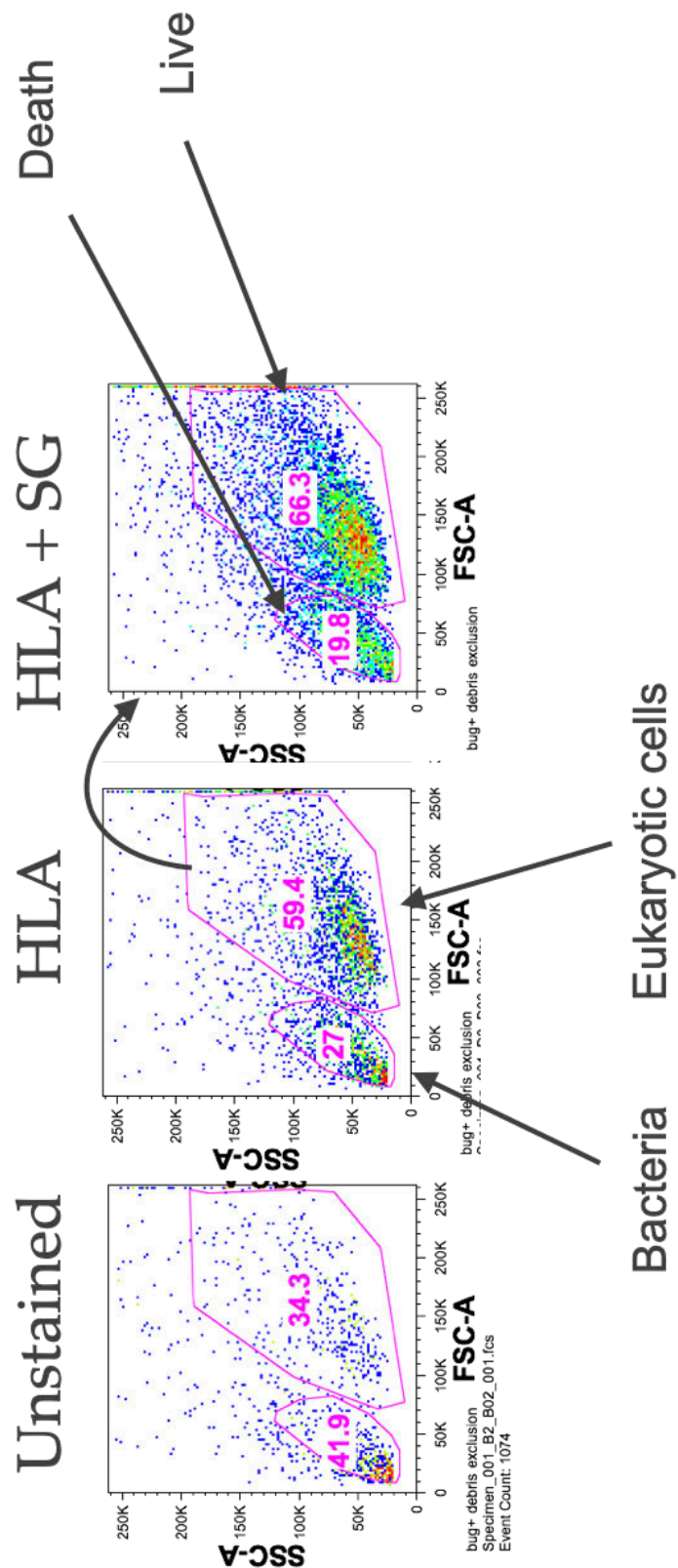


Figure 4.9. Example of result obtained after flow cytometry experiment

Cells unstained, stained with HLA (Human Leukocyte Antigen, therefore selecting eukaryotic cells) and stained with both HLA and SG (SybrGreen, assessing the viability of the cell)

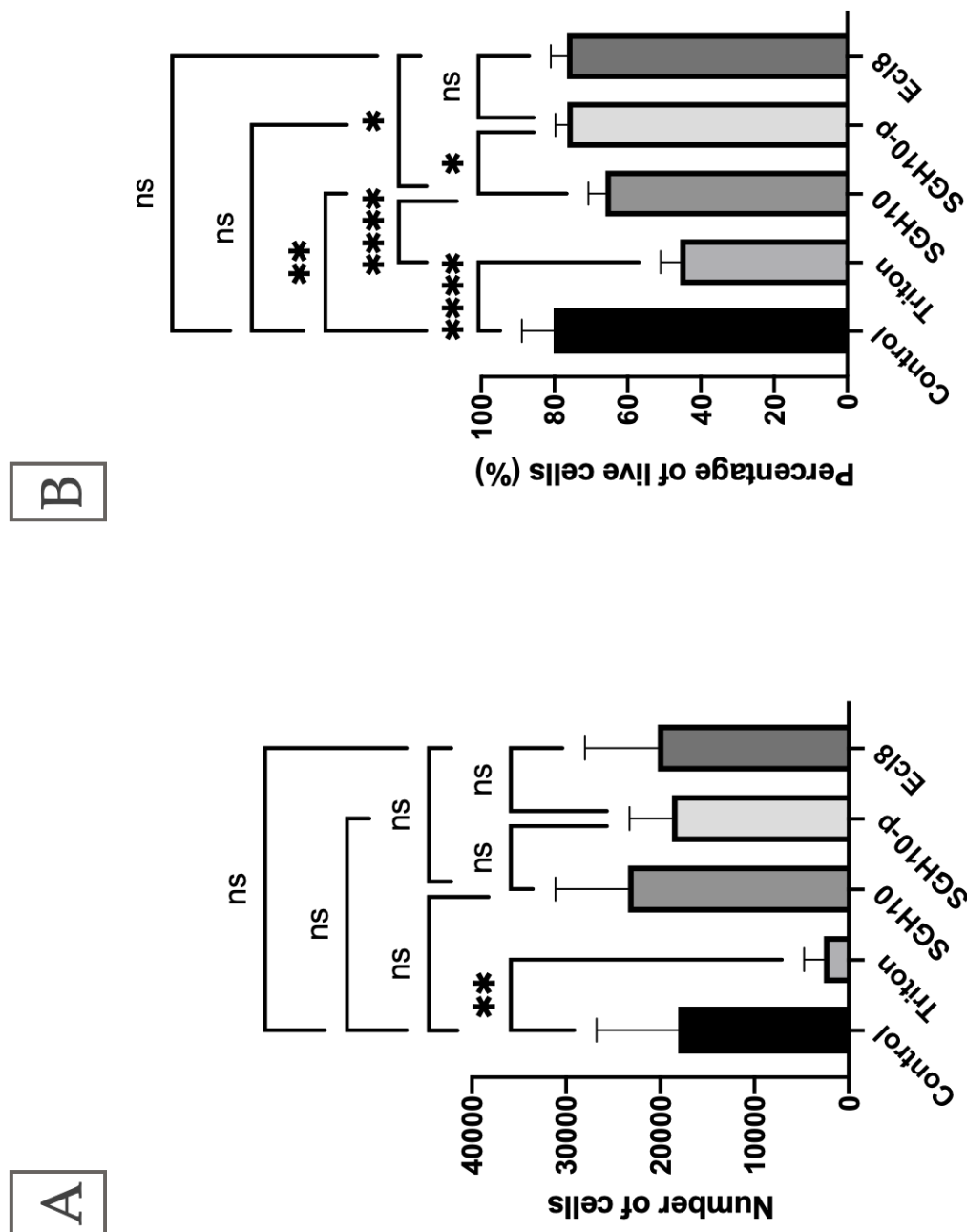


Figure 4.10. A. Absolute number of cells (dead or live) recovered for each condition, excluding debris. B. Percentage of live cells of the HepaRG cells measured by SybrGreen staining after acquisition with flow cytometry.

After no treatment (negative control), treatment with Triton (positive control), infection by SGH10, SGH10-p and Ecl8. Bars represent mean and standard deviation of three technical and biological replicates, compared with a one-way ANOVA test with multiple comparisons. ns = non-significant. *adjusted $p < 0.05$. **adjusted $p < 0.01$. ***adjusted $p < 0.001$. ****adjusted $p < 0.0001$.

4.3 Discussion

In this chapter, we successfully developed an ECIS-based model to study the host-pathogen interactions of HvKp. This model seemed relevant and robust, as it enabled us to highlight the differential effect on hosts cells depending on the capsulation and HMV levels of our strains.

Specifically, our main finding was the description, for the first time, of specific effects of HvKp on both intestinal and liver cells comprising of greater TJ disruption and cell membrane toxicity. These results relied on the use of $T_{50}DF$, which we considered to be the most informative and suitable technique for the quantification of bacterial toxicity using ECIS. For instance, some authors failed to demonstrate differences in virulence between different bacterial species (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus spp.*) using ECIS but quantifying changes by simply averaging all data points.²¹³

By looking first at the intestinal epithelium, modelled by the Caco-2 cell line, we found that SGH10 disturbed TJs significantly more effectively than SGH10-p and Ecl8. This suggested that SGH10 has the ability to cross this intestinal epithelium via the paracellular route. In the light of the genophenotypical characteristics of the three strains used (**Table 3.2**), the difference observed in the intensity of this effect between SGH10 and SGH10-p indicated that this effect on TJs is linked to the HMV of SGH10, and not to either siderophore production or capsulation. HMV contributes to the virulence of HvKp by reducing adherence/association with host cells, leading to both

avoiding phagocytosis and paradoxically improving persistence in the mouse gut.^{147,150,152}

Although the precise mechanics of HvKp toxicity on intestinal cells will be further investigated and discussed in the next chapter, this may already suggest that HMV, by preventing association with epithelial cells, forces the bacteria to stay around the cells and interact with their TJs, further passing through a paracellular route.

Interestingly, the only study to date looking at the interactions between Caco-2 and Kp *in vitro* concluded that Kp passes through this intestinal barrier by a transcellular route, engulfed into gut cells.²⁸⁸ However, three crucial factors differentiated this study from our work: (1) The strains used in this study were not HvKp strains (or at least not genomically defined as such), and no information on their level of capsulation or HMV was given, (2) The only quantitative results were obtained from the use of TEER, but with recording for only one hour. In contrast, effects were picked up when TEER recorded for 4 hours and (3) this data was supplemented by immunohistochemistry visual assays, but these were only qualitative and therefore of limited value.

The apparent contradiction between the results of this study and our own must therefore be put into perspective, especially since, as illustrated in the next chapter, cKp probably does use the paracellular route to pass through the intestinal epithelium.

Because HvKp can colonise the gut for years before causing a liver abscess, we believe that this passage through the intestinal epithelium is the key element in its infection process. Yet, the study of its interactions with liver cells is still relevant, and has hardly been carried out so far. Our ECIS results showed that SGH10 and SGH10-p differed from Ecl8 in that they were more toxic to liver cells. This increased toxicity of HvKp was confirmed using our flow cytometry method, which even revealed a

difference between SGH10 and SGH10-p. While this contradiction prevented us from defining a clear role for the capsule or HMV in this process, we could still conclude that HvKp (with or without a virulence plasmid) causes greater liver damage than a non-hypercapsulated and non-HMV Kp.

One limitation to drawing a conclusion is that another major difference between these strains that could explain the variation is that Ecl8 doesn't secrete colibactin. Colibactin is a genotoxin causing cell damage, however, "only" present in 80% of HvKp strains, which means that strains lacking colibactin can also cause abscesses.

The second and main limitation in interpreting these results is the absence of an immune system in our *in vitro* setting. Kupffer cells, liver-specialised macrophages acting as the first defence line when a pathogen reaches the liver from the portal system, have been described as important in HvKp pathophysiology, but in a contradictory manner. One study showed that they had increased and specific efficacy on HvKp both *in vitro* and *in vivo*²⁰⁵, while others suggested that HvKp had developed various mechanisms to escape them.^{289,290} Either way, these immune cells play an important role, and their absence in our model prevents us from drawing clear conclusions about the extent of HvKp interactions in the liver.

A final observation from our results is that these differential effects of HvKp are restricted to cells of the intestine-liver axis. The mechanisms explaining this exclusivity are not known and nothing comparable has been described in the literature. Yet, this suggests that HvKp has specific bacterial factors enabling them to interact preferentially with the cells they target during their pathogenic process, and could be the subject of future research.

In conclusion, this chapter shows that we have been able to develop an ECIS-based model demonstrating increased toxicity of HvKp on intestinal TJ and liver cell membrane. The next chapter will attempt to understand the dynamics of these toxic effects, as well as the mechanics of this TJ disruption.

Chapter 5: Further characterisation of the damage and disruption of epithelial cells in colonisation and pathogenesis by HvKp

5.1 Introduction

We developed our HvKp infection model using ECIS as it explores, amongst others, the effect of pathogens on host cells. This model allowed us to demonstrate for the first time HvKp's specific pathogenic action along the gut-liver route, on intestinal (greater TJ disruption) and liver (higher global toxicity) cells, ultimately leading to the formation of pyogenic liver abscesses. These HvKp-specific infectious effects were highly dependent on their HMV/hypercapsulated nature, but their precise mechanisms are unknown. We therefore aimed to use and adapt this model to clarify and characterise them.

The first question we addressed was whether the observed HvKp effect was dependent on live bacteria or secreted factor. This question arose from the fact that some intestinal pathogenic bacteria work by secreting a toxin (e.g. *Clostridioides difficile*), while others require the bacteria to be alive, for example to invade and survive in host cells (e.g. *Salmonella typhi*).^{60,279,280,291}

Secondly, we wondered if bacteria were internalised or if they had to remain in contact with the host cells to exert their toxic effect. Indeed, although Kp is classically known as an extracellular pathogen, its internalisation and survival in immune cells

(macrophages and neutrophils) and in intestinal cells have already been demonstrated.^{184,189,288,292–294}

Thirdly, we aimed to evaluate whether its toxic effect could be linked to an immune modulation phenomenon by assessing the levels of cytokines secreted by gut and liver cells after infection. As described in the general introduction, one of the main effects (and infection mechanisms!) of *HvKp* is to impair this immune response in order to survive. Yet, this did not rule out the possibility that some immunological responses specific to intestinal or hepatic cells might be triggered. *Salmonella enterica* (serovar Typhimurium), for instance, exploits the host's immune system by inducing the secretion of pro-inflammatory cytokines by immune cells but also by intestinal epithelial cells. These include interleukin-8 (IL-8), tumour necrosis factor alpha (TNF- α), and interferon-gamma (IFN- γ), and lead to the recruitment of neutrophils to the intestinal mucosa and the disruption of the gut barrier.^{295–297}

Next, because we have shown that the toxic effects of *HvKp* were specific to gut and liver cells, we wanted to determine whether *HvKp* instigated or affected the interactions between these two systems. Liver diseases such as NAFLD or cirrhosis are aggravated by toxins secreted by bacteria present in the intestine and transmitted via the portal vein.^{59,58} However, no description of communication between bacteria and this intestine-liver axis, in either direction, has been proposed to date. We therefore first looked for a factor secreted by the intestinal cells in response to infection by *HvKp*, further acting on the liver cells.

We then designed a Transwell system to (1) study the passage of *HvKp* through this intestinal cell layer, and (2) evaluate whether this had any influence on their subsequent effect on liver cells.

Because our results suggested that intestinal TJ disruption was critical in *HvKp*'s pathogenicity, we later explored the effect of *HvKp* on the different proteins comprising the TJ. Gut TJ proteins are indeed a critical component when studying intestinal bacteria pathogenicity. For instance, *Salmonella typhimurium* disrupts gut TJ by redistributing claudin-1, occludin, and E-cadherin junctional proteins.²⁹⁸ Other bacteria like *Campylobacter jejuni* will secrete outer membrane vesicles that possess a protease activity, cleaving occludin and E-cadherin.²⁹⁹

Finally, while the characteristics of our strains made us fairly confident that hypercapsule and HMV were the main microbiological properties of *HvKp* responsible for our observations, we sought to determine precisely which of the genes responsible for these phenotypes were necessary to these interactions. This was attempted by generating mutants of SGH10 lacking *rmpA*, *rmpC*, *rmpD* and *wcaJ*.

Overall, the aim of this chapter was to better understand and elucidate the mechanistic and interconnections of the specific effects of *HvKp* along the gut-liver axis.

5.2 Results

5.2.1 HvKp's toxicity on gut and liver cells necessitates live extracellular bacteria

To determine whether the detrimental effect of HvKp on gut and liver cells could be due to a secreted factor or live bacteria, we first simply incubated eukaryotic cells seeded on an ECIS plate with heat-inactivated bacteria. We specifically looked at 64kHz (cell membrane integrity) for HepaRG and 4kHz for Caco2 (TJ), since these were the conditions under which we were able to observe a specific effect of HvKp in the direct infection model.

As shown in **Figure 5.1**, the heat-inactivation of our strains prevented any harmful effect on either Caco-2 and HepaRG, with resistance values of bacteria-infected all staying above or around the cell-only control, despite an extended 24-hour monitoring timeframe.

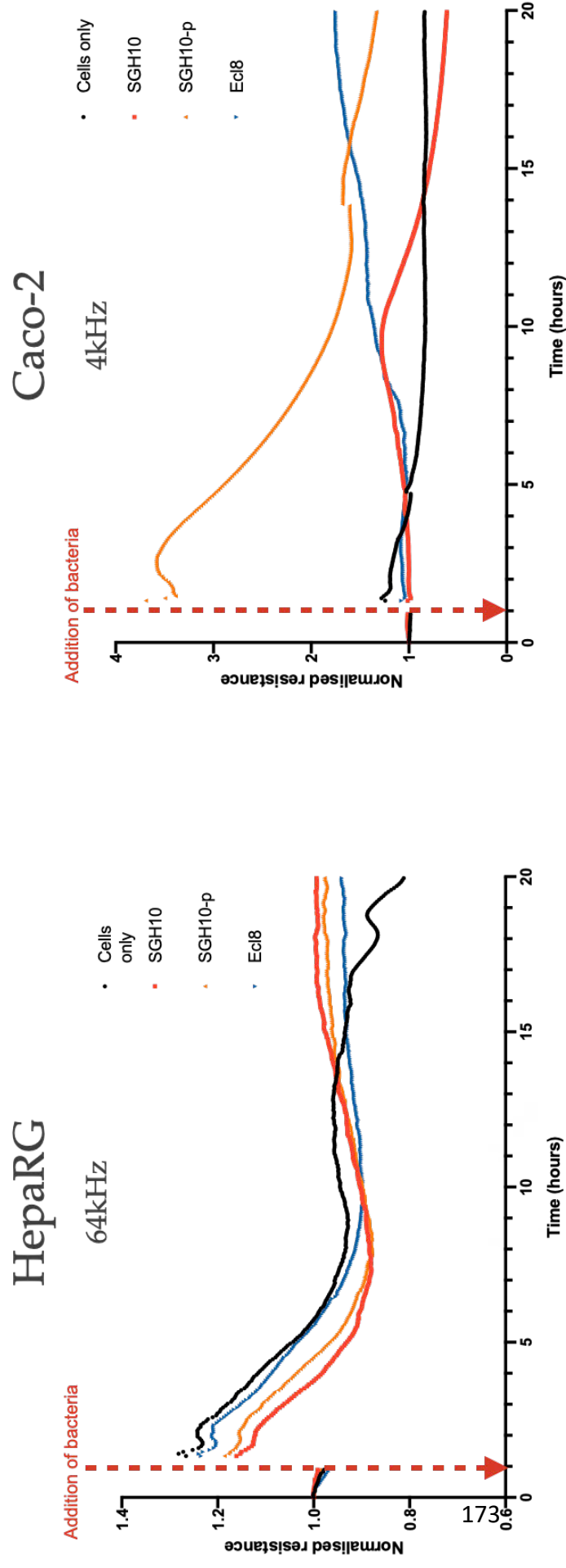


Figure 5.1. Normalised resistance of HepaRG (64kHz) and Caco2 (4kHz) cell layers monitored by ECIS over time, after addition of heat-inactivated bacteria.

Red dashed arrows indicating the time point at which the bacteria were added. No specific effect observed after bacterial infection for any of both graphs, as resistance values of bacteria-infected wells staying above or around the cells-only controls.

Three biological replicates with two technical replicates each.

We therefore designed a second experimental setting, which consisted of infecting our intestinal and liver cells as described above, but then removing the bacteria from the ECIS plate well after 2 or 4 hours, while replacing the supernatant free from bacteria (ensured by filtering the bacteria and adding antibiotics to ensure sterility).

The aim of this last assay was twofold: (1) to cross-check the presence of factor secreted by the bacteria (which should have remained in the supernatant, since it had not been removed by filtration or affected by the antibiotics), but also (2) to exclude the possibility that the effect observed was linked to the internalisation of bacteria into eukaryotic cells, thus not requiring their continuous presence in contact with the cells. As displayed in **Figure 5.2**, no damage was observed for any of our strains on HepaRG, or for SGH10 and SGH10-p on Caco-2. In contrast, Ecl8 still exerted a deleterious effect on Caco2's when the bacteria were removed after 4 hours (see curve highlighted in yellow). This could be easily explained by the fact that Ecl8 has been shown to be significantly more internalised by Caco2 than the other 2 strains. Yet, we could conclude that the specific effect of HvKp (1) is not due to the secretion of a factor, as neither heat-inactivated or bacteria-free supernatant were causing any harm, (2) is probably not the consequence of its internalisation in eukaryotic cells, and therefore (3) necessitates the presence of live extracellular bacteria.

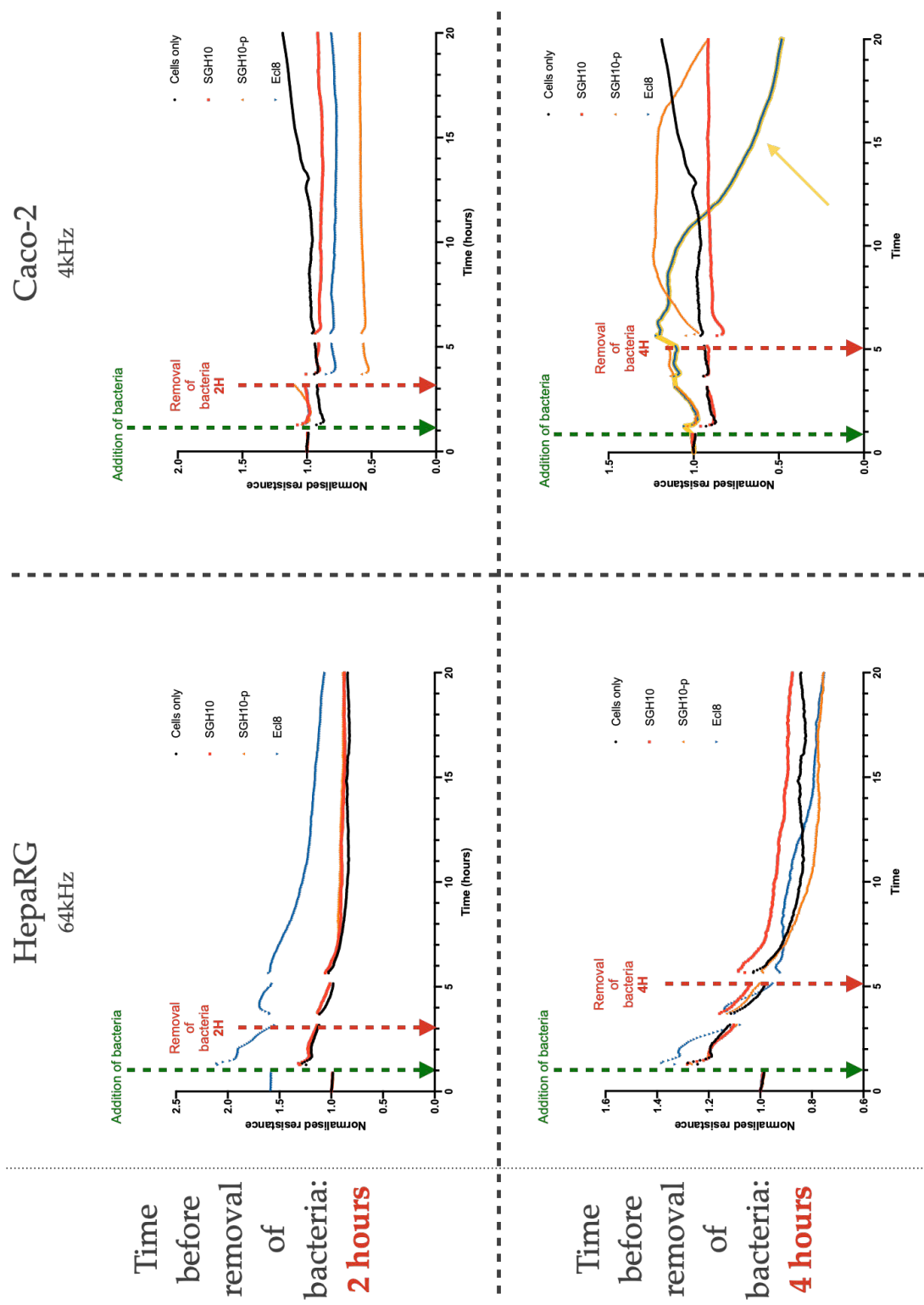


Figure 5.2. Normalised resistance of HepaRG (64kHz) and Caco2 (4kHz) cell layers monitored by ECIS over time, after removal of bacteria from supernatant after 2 hours (upper graphs) or 4 hours (lower graphs).

Green dashed arrows indicating the time point at which the bacteria were added. Red dashed arrows indicating the time point at which the bacteria were removed from the well.

No specific effect observed after bacterial infection as resistance values of bacteria-infected wells staying above or around the cells-only controls, except from persisting harmful effect of Ecl8 on Caco-2 cells after 4 hours incubation indicated by a yellow arrow (lower right panel).

Three biological replicates with two technical replicates each.

5.2.2 Assessment of eukaryotic cell cytokine secretion

After investigating various microbiological factors as potential explanations for the specific effect observed with *HvKp*, we wanted to check that this effect was not due to a difference in the immune response of the intestinal or liver cells themselves. With this in mind, we measured the secretion of cytokines by HepaRG and Caco-2 in response to infection with our different strains.

These results are shown in **Figure 5.3**. Their interpretation was limited by the fact that the levels of cytokines secreted by these cell lines were low, preventing the mathematical extrapolation of the sample concentration from the PE (PhycoErythrin, fluorescence) intensity using standard curves for any of our samples except from IL-8 secretion by the HepaRG cells. This might explain that overall we were unable to identify any significant differences in cytokine secretion between our different strains, either for Caco-2 or for HepaRG.

Yet, while trend analysis of the values showed strictly no difference for Caco2, we were able to observe higher secretion of IL-10 (anti-inflammatory cytokine) and lower secretion of IL-8 (pro-inflammatory cytokine) in response to infection of HepaRG by SGH10. While this was not described before but was consistent with the known anti-inflammatory effect of *HvKp* (developed in section 1.5.6 and illustrated with our results in Chapter 3), it probably does not explain the differences observed in terms of cell membrane toxicity seen on ECIS (see discussion in section 5.3).

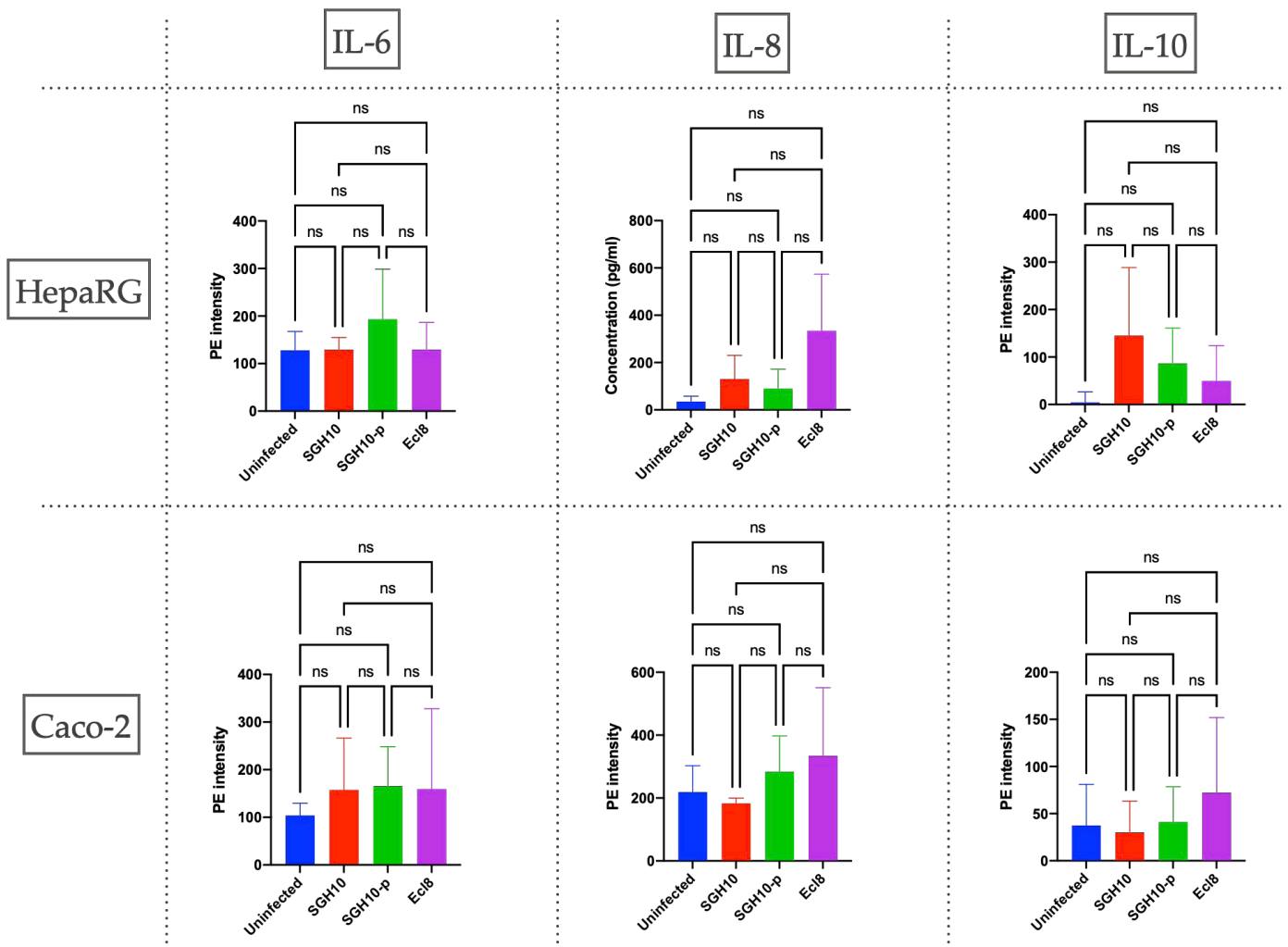


Figure 5.3. IL-6, IL-8 and IL-10 secretion after infection of Caco-2 or HepaRG with SGH10, SGH10-p and Ecl8.

All results expressed as the PE fluorescence intensity, except for the HepaRG cells IL-8 secretion, expressed as concentration. No significant differences between strains observed in terms of cytokines secretion from either HepaRG or Caco-2 cells.

Three biological replicates with two technical replicates each. ns = non-significant.

5.2.3 Characteristics and communication of HvKp infection along the gut-liver axis

5.2.3.1 *Incubation of HepaRG with supernatant of infected Caco2*

We aimed to establish the relationships, if any, between the intestine and the liver during HvKp infection process, given that HvKp translocate from the intestine to the liver. As a matter of fact, we recently demonstrated such gut-liver communication in the case of paracetamol, showing the effect of supernatant from treated Caco-2 transferred to HepaRG is different from the direct exposure of HepaRG to paracetamol.³⁰⁰

With this in mind, we first examined the possibility that the infection of intestinal cells with HvKp would trigger them to secrete a factor acting on HepaRG. In order to verify this, we infected Caco-2 with the strains panel, then collected the supernatant, which was sterilised by filtration and addition of kanamycin and finally transferred to HepaRG. As **Figure 5.4** illustrates, we did not observe any deleterious effect on liver cells, either in terms of cell integrity (64kHz) or TJ disruption (4kHz), with no decrease of resistance after incubation with the post-infection supernatant when compared to the control line. This ruled out Caco-2 secreted factors as a mediator of toxicity HepaRG triggered by HvKp infection.

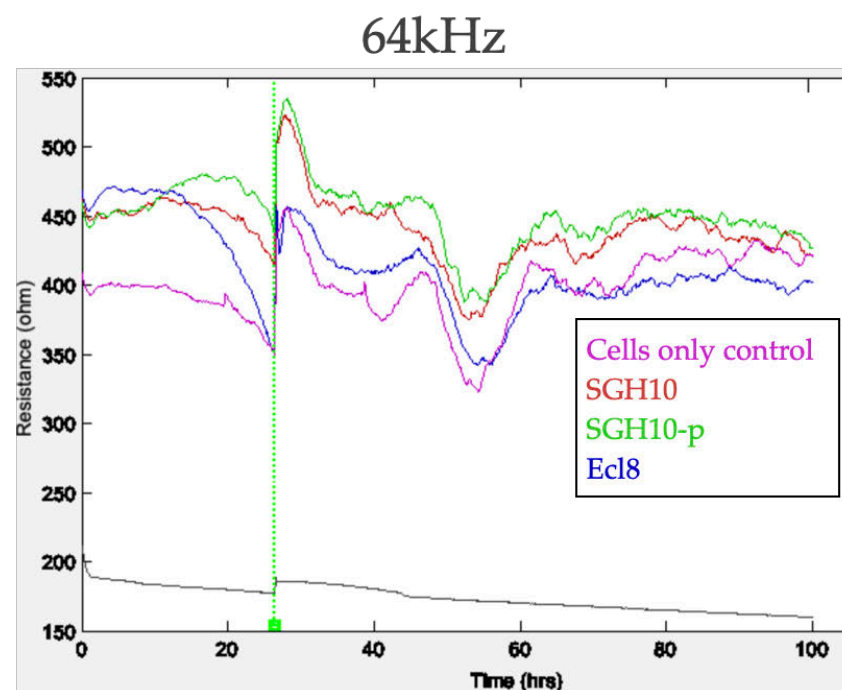
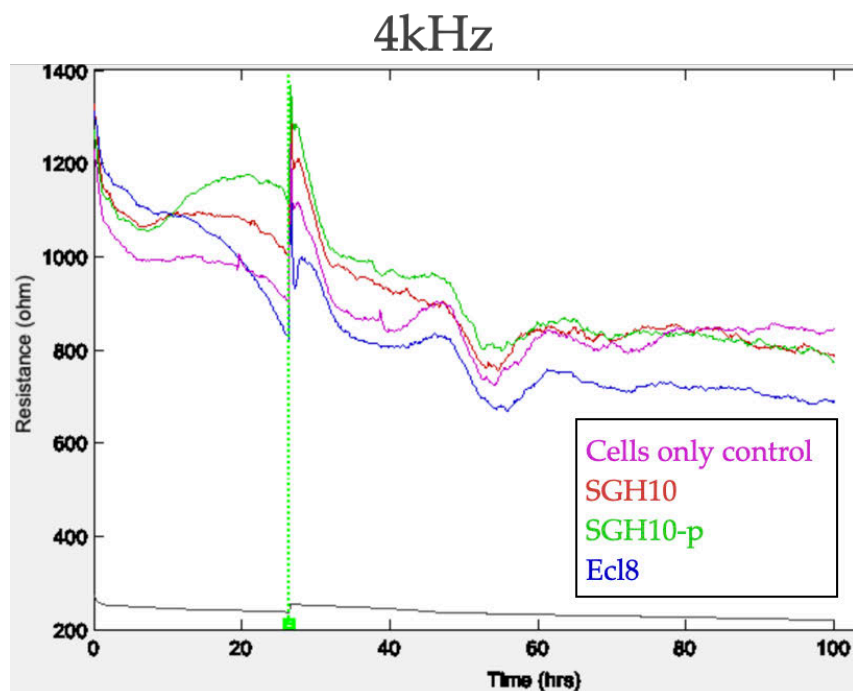


Figure 5.4. Assessment of TJ (4kHz) and cell membrane integrity (64kHz) of HepaRG cells after incubation with supernatant from infected Caco2's, cleared of bacteria

Vertical dashed green line: time of addition of bacteria.

Single biological replicate, two technical replicates.

5.2.3.2 Transwell system on ECIS

We next attempted a model mimicking the gut-liver communication axis by devising a 2-stage Transwell system. An insert coated with Caco-2 cells was connected to an ECIS well seeded with HepaRG (see Figures **2.4.B** and **5.5**). This setup was technically difficult to create, as we needed to ensure that the two well were correctly separated by the membrane, but also that there was no gap between any component that could artificially hinder bacterial progression. We achieved this by developing 3D printed connectors between the inserts and the ECIS wells, which attached perfectly to the 2 components, preventing any media leakage (see **Figure 5.5**).



Figure 5.5. Transwell setup on ECIS, showing inserts into 3d-printed connectors, further introduced into an ECIS plate well.

Prior to using this system, we needed to determine the rate at which our 3 strains passed through the Caco-2 cell layer in the insert. This was established by adding bacteria to the upper well of a Transwell insert and assessing their presence in the lower well over time. As shown in **Figure 5.6**, Ecl8 was rapidly found in the lower well, as early as 1/2 hour after its addition to the upper well, unlike SGH10 and SGH10-p, which were only found in the lower well after 4 hours of incubation (therefore taking 8 times longer to cross the layer). The most plausible hypothesis is that Ecl8 uses the transcellular route as we also have previously shown that Ecl8 is internalised in Caco2. Conversely, the delay in SGH10's passage through Caco-2 cells, along with the fact that it poorly adheres and is not internalised in gut cells, probably indicated that it uses the paracellular route. This was also suggested by our ECIS results which shows that SGH10 disrupts the TJs, key elements for epithelial cells to prevent the paracellular passage of bacteria.

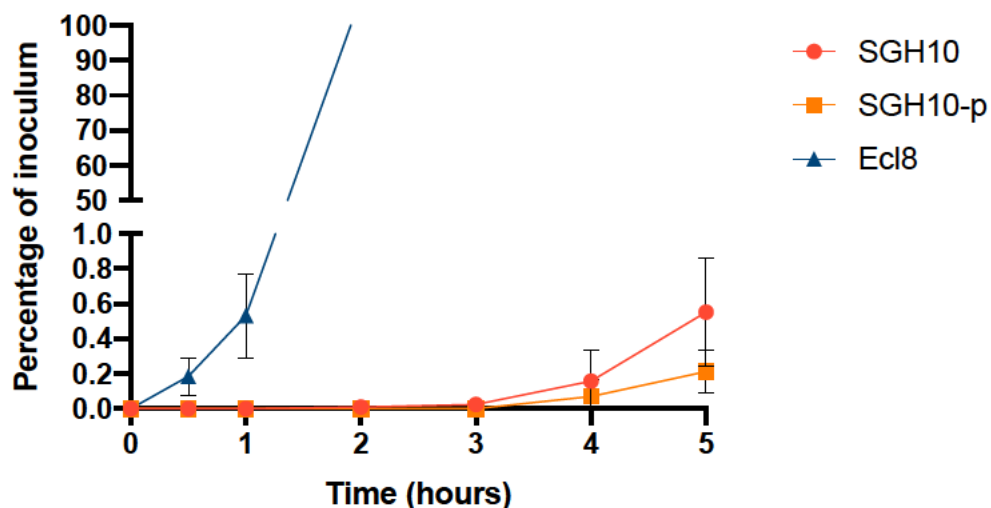


Figure 5.6. Percentage of the inoculum retrieved in the lower well of our Transwell system over time.

Bars represent standard error of three biological replicates.

Interestingly, the analysis of the entire system revealed that, despite having to first pass through Caco-2, SGH10 retained an overall toxicity on HepaRG, with a T_{50DF} of around 10 hours. As illustrated in **Figure 5.7**, this T_{50DF} was almost similar to that observed when they were inoculated onto HepaRG directly (just under 8 hours). However, this was not the case at all for Ecl8, which lost virtually all toxicity at both TJ and cell membrane level after passing through the intestinal cells, with no decrease in resistance.

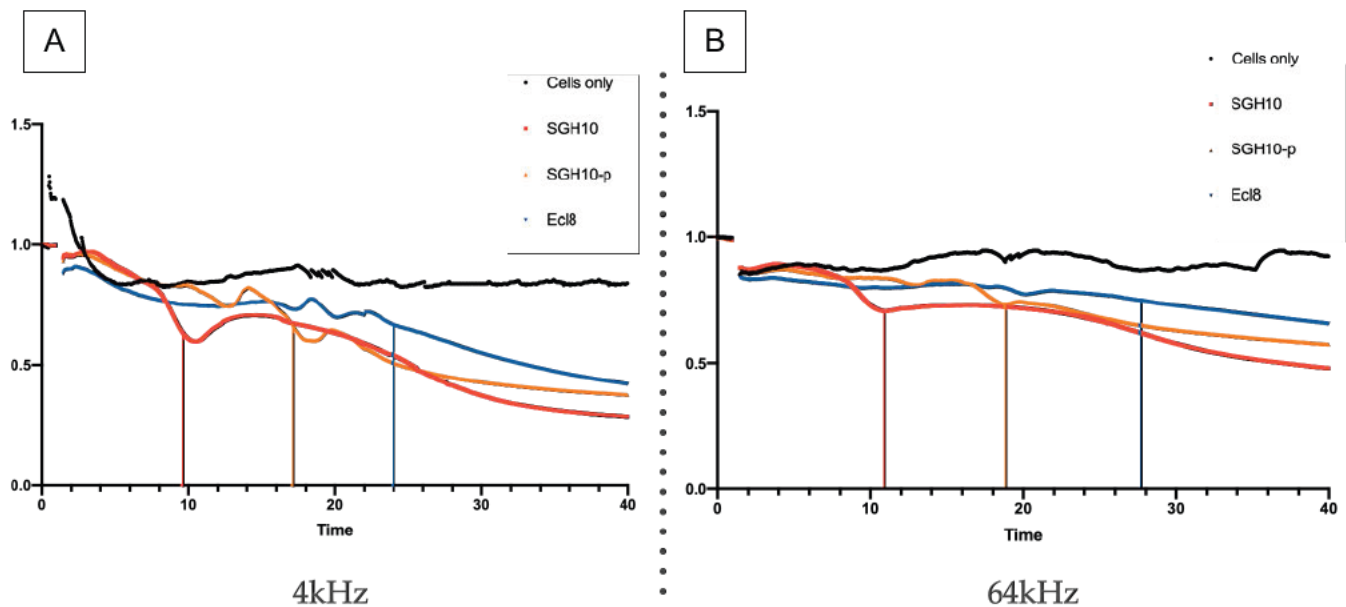


Figure 5.7. Determination of T_{50DF} of SGH10, SGH10-p and Ecl8 on HepaRG cells A. TJ (4kHz) and B. cell membrane integrity (64kHz), after passage through the Caco-2 layer seeded in Transwell insert.

Average values of three biological replicates and two technical replicates for each experiment.

5.2.4 Particular effect of HvKp on intestinal TJ proteins

Our ECIS results strongly suggested that the mechanism by which SGH10 passed from the intestines to the portal system was by disrupting gut epithelial cells' TJs. We thus set out to determine exactly how this happened. While the analysis of the redistribution of these proteins often requires more extensive and convoluted experiments, their degradation can be established by measuring their levels. This was done by immunoblotting, specifically targeting ZO-1, claudin-1, claudin-3 and occludin. These proteins were chosen as claudins and occludin are the two main proteins expressed in the intercellular space responsible for TJ integrity, and ZO-1 is the critical factor linking these extracellular proteins to the cytoskeleton.^{17,301,302} Also, they are by far the most involved in the pathophysiological mechanisms of bacteria targeting the TJ of the intestinal epithelium.⁴⁸

Proteins were extracted from Caco-2 cells, after a 3-hours infection with our strains panel. After quantification of the total protein levels, we specifically probed the TJ proteins. **Figure 5.8** shows the expression levels of these, derived from the signal intensity of the immunoblotted bands, normalized to GADPH expression, relative to the control (uninfected cells). EGTA was used as a control, since it is a calcium chelator, uncoupling but not destroying TJs, therefore not decreasing their levels. Of note, this experiment required a considerable amount of troubleshooting, particularly in terms of the antibodies used, the type of lysis buffer applied, the quantity of eukaryotic cells to be infected to obtain a sufficient quantity of proteins and the exposure time during image development. We initially used the radioimmunoprecipitation assay (RIPA) lysis buffer, with a very low protein yield. We therefore switched to the MRC lysis buffer (see

Table 2.8), with higher levels, enabling us to probe and visualise them. However, the antibodies initially used for ZO-1 and claudin-1 did not work and had to be changed to those specified in section 2.5. Also, we initially used 12-well plates, but we realised that there were too many proteins, which reduced the sensitivity of our test and prevented us from discriminating between the effects of different bacteria on TJ in Caco-2. This was resolved by using 96-well plates. Finally, the optimal exposure times varied greatly between the different proteins and were therefore difficult to determine. After this fine-tuning process, the results showed that there was no difference in terms of ZO-1 or claudin-3 expression after infection with our different bacteria. By contrast, the expression of occludin and claudin-1 was significantly reduced after infection with SGH10 compared with both the control and the other two bacteria. This was particularly marked for claudin-1, with a more than two-fold reduction in expression compared with the control (**Figure 5.8**).

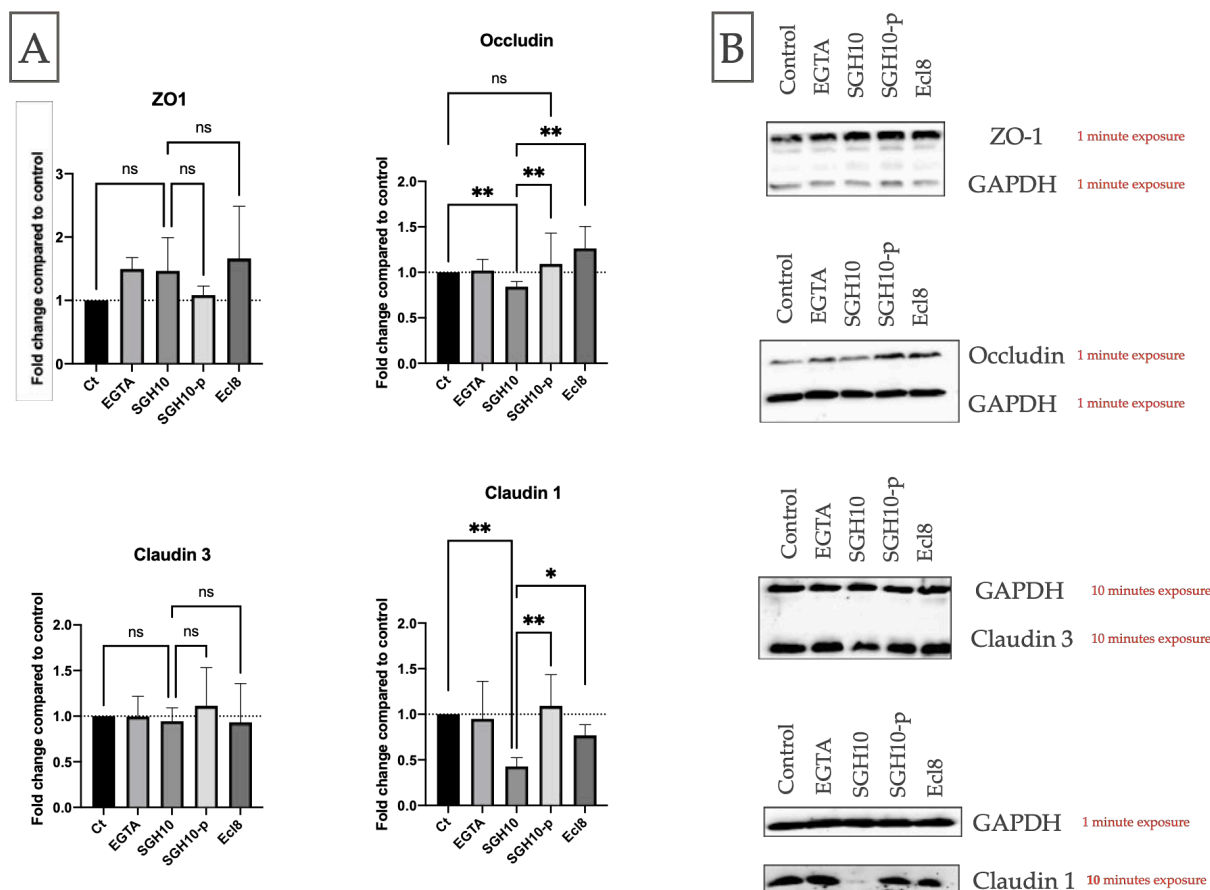


Figure 5.8. Western Blot results for TJ proteins levels after incubation with EGTA or infection with SGH10, SGH10-p and Ecl8.

A. Fold change of the band intensity after 1-minute exposure (except for claudin-1, 10 minutes), normalised to GAPDH and compared to the Ct (Control) band (media only). Bars represent mean and standard deviation of three technical and biological replicates, compared with a one-way ANOVA test with multiple comparisons. ns = non-significant. *adjusted $p < 0.05$. **adjusted $p < 0.01$. B. Illustration of the band obtained for occludin (upper panel) and claudin-1 (lower panel).

5.2.5 Role of HvKp's specific HMV and hypercapsule genes to this pathophysiology

Finally, we wanted to assess the precise role of each of the various genes involved in the development of the HMV and hypercapsulated HvKp phenotype in its pathophysiology at intestinal and hepatic level. To this end, we aimed to create chromosomal mutants of these genes: *mpA*, *mpC*, *mpD* and *wcaJ*, using the allelic exchange technique. For each of these genes, we succeeded in generating an exchange vector plasmid (pTOF25) comprising the downstream and upstream flanks of the chromosomal gene of interest, itself replaced by the kanamycin cassette. Introducing this plasmid into SGH10 was challenging but achieved using an adapted electroporation protocol as described in the materials and methods section. Presence of plasmid was confirmed by PCR. Unfortunately, despite PCR confirmation of the presence of the plasmid in our strain, our numerous attempts at allelic exchange have all failed, stalling at various stages in the process. Yet, this work is still underway.

5.3 Discussion

Having used our ECIS-based infection model to demonstrate *HvKp*-specific interactions at the intestinal and hepatic levels, all the data presented in this chapter enabled us to propose a general mechanism of formation of pyogenic liver abscess by *HvKp*.

Firstly, although these interactions are specific to the gut-liver axis, the reason for this specificity is unclear, as it did not appear in our results that *HvKp* influences or makes use of the communication between these two organs. To our knowledge, no other pathogens with specific and exclusive interactions with intestinal and liver cells have been described.

Secondly, while no difference in terms of cytokines secretion by Caco-2 after infection with the different strains was noticed, the infection of HepaRG by the similarly hypercapsulated SGH10 and SGH10-p lead to lower levels of inflammatory cytokines and higher levels of anti-inflammatory cytokines when compared to Ecl8.

With the limitation that no specific immune cells were present, these results are in line with the anti-inflammatory effect of *HvKp*'s hypercapsule, described in section 1.5.6. However, since we have demonstrated greater toxicity of SGH10 and SGH10-p on these hepatocytes, these cytokine secretion results are somewhat contradictory. We therefore concluded that no immune phenomenon is involved in this pathogenicity.

Thirdly, the results obtained using our Transwell model were particularly interesting: We previously shown in this work that *HvKp* were not internalised in Caco-2 cells but

disrupted their TJ. In light of this information, the demonstration in our Transwell model of a delayed passage through this gut epithelial barrier suggested that they had no other choice than to pass via the paracellular pathway.

Ecl8, which does not express the HMV phenotype, gets internalised in the Caco-2 cells, passing through intestinal epithelium via the transcellular pathway. These findings confirm what Hsu et al. have demonstrated with other *cKp* strains.²⁸⁸

Interestingly, this internalisation allows *cKp* to cross the epithelium faster but also appeared to impair their ability to further harm liver cells. By extrapolation, this suggests that the avoidance of internalisation and subsequent paracellular passage of *HvKp*, provided by its HMV and hypercapsule, contributes to its overall virulence by preventing that decrease in toxicity observed in *cKp*.

This progression of *HvKp* through the paracellular pathway is supported by the disruption of tight junctions. In this context, the decrease of claudin-1 and occludin triggered by SGH10 was noteworthy, as they have been shown as the critical proteins for TJ barrier function integrity.^{301,303,304}. Claudin-1 is for instance reduced in inflammatory bowel diseases^{305,306}, whereas claudin-3 is only expressed in the colon and has a less clear role in barrier function.^{307,308} The lack of effect on ZO-1 expression was expected as this is an intracellular scaffolding protein, connecting claudin and occludin to the cytoskeleton, therefore not in contact with *HvKp*.

Finally, because we were able to show that no toxin was playing a role in these observed effects and that SGH10-p, which did not secrete aerobactin, retained a greater toxic effect than Ecl8 on both Caco-2 on HepaRG, we concluded that the HMV

and/or hypercapsulated nature of HvKp were the main factors responsible for its specific pathophysiological process. This was somewhat surprising, as aerobactin has been shown to increase HvKp virulence in mouse systemic and pulmonary infection models.^{156,157}

Overall, we postulate that HvKp's infection process, illustrated in **Figure 5.9**, is as follows: Viable HvKp, on reaching the intestinal cells, has to remain external and alive to allow it to disrupt the tight junction proteins claudin-1 and occludin by directly interacting with them, to further perturb the integrity of the paracellular pathway which it will then use to enter the portal system. Once in the liver, we demonstrated that HvKp has an increased toxicity on liver cells, further promoting abscessation by promoting hepatocytes' killing.

This mechanism, based on our *in vitro* model, is novel and has never been described before for HvKp. However, *E. coli* K12, usually considered a commensal of the intestine, has also been shown to cause intestinal infection and extraintestinal tissue invasion *in vitro*, linked to a toxicity on tight junctions, particularly on claudin-1 and occludin.³⁰⁹ The striking similarity between these two mechanisms reinforces the plausibility that HvKp does indeed use this pathway to switch from colonising the intestine to creating an abscess in the liver. The microbiological characteristic that enables HvKp to cause this damage to tight junctions remains unclear, but when comparing SGH10, SGH10-p and Ecl8, we can confidently state that HMV is the most crucial factor in that crossing of the gut, and both HMV and capsule are important in HvKp's liver toxicity. A final confirmation would have been obtained if we had succeeded in developing mutants of genes specific for these phenotypes.

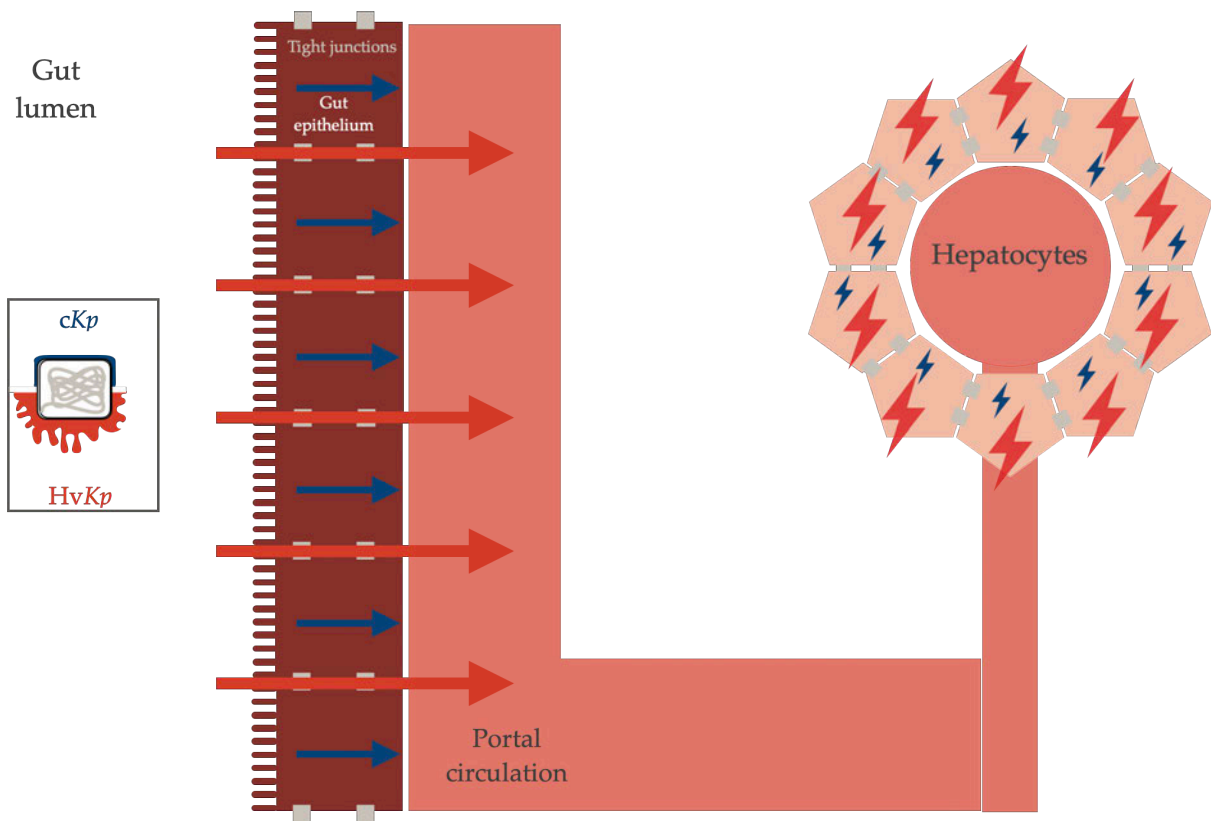


Figure 5.9. Schematic representation of *HvKp*'s pathophysiological mechanism to switch from coloniser in the gut to pathogenic in the liver.

Chapter 6: General discussion

Whilst *Klebsiella pneumoniae* was described at the end of the 19th century, the discovery of its hypervirulent variant is much more recent, dating from to the 1980s. HvKp causes a distinct clinical syndrome, comprising of pyogenic liver abscess and dissemination to deep tissues in healthy, young and immunocompetent individuals. To date, the phenotypic and genotypic attributes most certainly defined as relevant to liver abscess formation are a thick capsule, the hypermucoviscous phenotype, the secretion of siderophores and the genotoxin colibactin.^{82,310,311} Yet, their precise and individual role and connectivity is unclear, as some strains will cause liver abscesses in healthy patients without possessing all of these phenotypes, and vice versa. Overall, the general infection process of HvKp is also poorly elucidated, but it is known that it causes these liver abscesses via the portal route, following colonisation of the intestine.

The aim of this work was to elucidate HvKp's pathophysiology by observing and describing its interactions with the human host eukaryotic cells, alongside the identification of which virulence factors are relevant in this process.

These three strains, differing in terms of mucoviscosity, capsule production and siderophore production, are summarised in **Table 3.2**, and consisted of SGH10 (hypercapsulated, HMV and secreting siderophores), SGH10-p (hypercapsulated, not HMV, no siderophore secretion) and Ecl8 (neither HMV nor hypercapsulated, but secreting siderophores).

The differences between these strains in terms of interactions with cells of the gut-liver axis (Caco-2 (gut), HepaRG (liver) and HUVECS (endothelial) cells) were investigated

by running them in different assays, including adhesion assays, internalisation assays, zebrafish larvae infection assays, but mainly an ECIS-based infection model assay that we developed for this work.

This model showed that SGH10 specifically disrupted the intestinal tight junctions more rapidly relative to SGH10-p and Ecl8, while SGH10 and SGH10-p both had a greater detrimental effect on the hepatocytes' membrane when compared to Ecl8.

These results, confirmed using alternative methods, prompted us to analyse intestinal TJ proteins' expression, showing that SGH10 specifically and significantly downregulated claudin-1 and occludin expression, two proteins critical for epithelial integrity.

The use of this model further allowed us to demonstrate that these SGH10 effects were specific to the gut-liver axis, required the presence of living bacteria, and that they were not linked to secreted factors or to the epithelial cells' immune response.

Finally, a Transwell system shed some light on the interconnections of this infection, confirming that *HvKp* was crossing the intestinal barrier more slowly than Ecl8, but that this enabled it to retain intact toxicity on the subsequently encountered liver cells.

Overall, the development of this model enabled us to describe a mechanism of action for SGH10 in its journey from coloniser in the intestine to abscess creator in the liver (**Figure 5.9**): As a result of the conjunction of (1) its low adhesion and absence of internalisation in gut cells and (2) its ability to disrupt gut cells tight junctions by affecting occludin and claudin-1, SGH10 passes through the intestinal epithelium and reaches the portal circulation using a paracellular route. Once in the liver, it presents

with a higher toxicity than Ecl8, potentiated by this lack of internalisation in intestinal cells, which appears to reduce virulence.

While *cKp* remains the most threatening form of *Kp* in terms of global public health, causing numerous opportunistic infections worldwide and associated with high rates of antibiotic resistance, *HvKp* also represents a growing problem. Although still most commonly readily treatable with antibiotics, the number of cases is increasing worldwide,^{101–104} and this infection leads to high mortality rates.⁹⁶

Yet, no mechanism of infection has been proposed in the literature to date. The current data is limited to the observation that *HvKp* colonises the gut before causing a liver abscess. The development and use of the clear-cut and powerful ECIS platform have enabled us to gain a much deeper understanding of the nature of *HvKp*'s interactions with human cells, and ultimately to propose for the first time a potential infection mechanism specific to *HvKp*. While this will have to be confirmed with *in vivo* experiments (as developed in chapter 7), it represents a significant advance in our understanding of this bacterium.

By using the three *Kp* strains described previously, we were able to establish that the hypercapsule and HMV were the two most crucial virulence factors in these *HvKp*-specific interactions.

HvKp's HMV nature was primarily responsible for reduced adhesion and internalisation and the destruction of intestinal TJs, therefore representing the key factor in the translocation of *HvKp* across the intestinal wall.

The molecular mechanism by which HMV causes the destruction of gut TJs has yet to be elucidated. Intuitively, we can hypothesise that a thick and viscous structure coming in contact with finely organised protein complexes can disrupt them by altering their

environment and affecting their organisation and dynamics, but this is not demonstrated. Another theory could involve the fact that the bacterial capsule is negatively charged. TJ proteins are particularly sensitive to the charge of molecules, as demonstrated by the charge-selectivity they provide to the epithelium, limiting the paracellular passage.^{312–314} While tight junctions proteins typically has regions with both positively and negatively charged amino acids,³¹² one could hypothesise that the hyperabundance of a negatively charged hypermucoviscous capsule could lead to deleterious interactions with these TJ proteins.

The capsule is not to be neglected since, as well as being essential for the generation of this HMV, the similar harmful effects observed for SGH10 and SGH10-p in the virulence in the zebrafish model and in the damage on hepatocytes showed that it played an equal part with HMV in these processes. The precise mechanism explaining how these two phenotypes commonly participate to virulence and affect the hepatocyte is also to be determined. The capsule allows *Kp* to avoid the local immunity mainly by masking surface antigens, mimicking human glycans and by repelling macrophages and neutrophils with its negative charge.^{289,315} As developed in section 5.3, the fact that the capsule provides for immune evasion probably indicates that its toxicity is not due to an inflammatory response. At the intestinal level, this also argues against a 'leaky gut' hypothesis, precisely due to systemic or local inflammatory activation.³¹⁶

Finally, the role of siderophores (in particular aerobactin) and colibactin were not demonstrated, therefore either non-existent or at least negligible compared with the role of HMV and the capsule.

Chapter 7: Limitations and future work

Although specific limitations have already been detailed in the discussions of each chapter, our work also presents more general limitations.

Firstly, although the impact of HMV and hypercapsule in our strains was strongly suspected 'phenotypically', we were unable to create mutants of the precise genes responsible for these phenotypes in a single strain. Other differences between the strains used in our model of which we would not be aware could therefore also theoretically explain these differences.

Secondly, we used a simple *in vitro* system, devoid of the microbiome and immune cells. We were therefore able to observe the 'raw' bacterial-epithelial interactions, but we lack information about the role of these other components present *in vivo*. In addition, we used cell lines that we considered relevant to HvKp's infection process but did not test our findings on primary human cells.

Finally, we used SGH10 because it was recently described as the archetypal HvKp strain, but Kp is unique in that most (more than 60%) of its genome is made of accessory genes, i.e. not present in all strains. There are therefore a huge number of differences between strains, with many regulators that may act in different ways, and it is therefore difficult to extrapolate our findings to all HvKp.

The future research that we could imagine for this work is therefore (1) to produce these mutants for the genes responsible for the hypercapsule and HMV (in progress),

(2) to test our strains and the mutants in an *in vivo* model that will enable us to take a close look at intestinal TJs (murine, for example), (3) depending on the results, to try to test molecules that will either reduce this HMV character or the HvKp capsule, or strengthen intestinal TJs, to counteract this mechanism in the human host and (4) to develop a vaccine targeting HvKp's surface antigens.

Other than antibiotics, no specific therapy against HvKp has been described to date, but many diet components such as polyphenols, oligosaccharides, short-chain fatty acids, zinc and vitamin D have been shown as enhancing TJ function.⁴⁵ One could therefore think that promoting a diet rich in these components in patients colonised by HvKp could limit the progression of the disease to a liver abscess. This could for instance be the subject of a randomised controlled trial, comparing the rate of development of liver abscesses in patients colonised by HvKp between those on a diet rich in these compounds and those on a normal diet. Of course, we are still a long way from achieving this, but this work provides an initial insight into the pathophysiological process of HvKp along the gut-liver axis, which is still largely unknown.

Finally, the demonstration of the crucial role of these elements present on the outer surface of the bacteria also raises the question of the vaccine. Since the capsular serotypes associated with HvKp are limited (essentially K1 and K2) and constant, these strains are ideal candidates for a vaccine.³¹⁷ This could eradicate their presence in the intestine and prevent the onset of the pathophysiological process we have described, but is not developed to date.

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Appendix

Primer	Description	Sequence
No_WcaJ_Smal	Forward primer for the generation of NoNi fragment for <i>wcaJ</i> deletion	aaggaaaaaa gcccgggc AGACAAAGTATCCACTATATC
Ni_WcaJ_NotI	Reverse primer for the generation of NoNi fragment for <i>wcaJ</i> deletion	cggaattc gcggccgc tgcagaac GTTCAATATTTTAAGGAAATCTTC
Ci_WcaJ_NotI	Forward primer for the generation of CoCi fragment for <i>wcaJ</i> deletion	gttctgca gcggccgc gaattccg TAAGATGCTGCTTAAGATAAG
Co_WcaJ_Sall	Reverse primer for the generation of CoCi fragment for <i>wcaJ</i> deletion	cgcacgcat gtcgac TGATAACCGAGTCCGTATTTG
No_RmpC_Smal	Forward primer for the generation of NoNi fragment for <i>rmpC</i> deletion	aaggaaaaaa gcccgggc AAAGATCTCGATCCGGAATCAGT
Ni_RmpC_NotI	Reverse primer for the generation of NoNi fragment for <i>rmpC</i> deletion	cggaattc gcggccgc tgcagaac AAAATTGTTATCTACACTATATTT
Co_RmpC_NotI	Forward primer for the generation of CoCi	cgcacgcat gtcgac GATCCTTCACATCCCCTCCCC

	fragment for <i>rmpC</i> deletion	
Ci_RmpC_Sall	Reverse primer for the generation of CoCi fragment for <i>rmpC</i> deletion	gttctgca gcggccgc gaattccg AGCTAACCACGTGGTTCCTCCCAAGT
No_RmpA_SmaI	Forward primer for the generation of NoNi fragment for <i>rmpA</i> deletion	aaggaaaaaa gcccgggc TTTACCCATGCGATAATGATT
Ni_RmpA_NotI	Reverse primer for the generation of NoNi fragment for <i>rmpA</i> deletion	cggaattc gcggccgc tgcagaac ACATCCCCTCCCCTTTTTTAC
Ci_RmpA_NotI	Forward primer for the generation of CoCi fragment for <i>rmpA</i> deletion	gttctgca gcggccgc gaattccg AGAAACAGTAACTTTGATCCA
Co_RmpA_Sall	Reverse primer for the generation of CoCi fragment for <i>rmpA</i> deletion	cgcacgcat gtcgac TGGTGATAAGGTAAGGCATAA
No_RmpD_SmaI	Forward primer for the generation of NoNi fragment for <i>rmpD</i> deletion	aaaaa gcccgggc TTTACCCATGCGATAATGATT
Ni_RmpD_NotI	Reverse primer for the generation of NoNi	cggaattc gcggccgc tgcagaac AGTATAGTTCTATAATAGTTTGAA

	fragment for <i>rmpD</i> deletion	
Ci_RmpD_NotI	Forward primer for the generation of CoCi fragment for <i>rmpD</i> deletion	gttctgca gcggccgc gaattccg CAAAAAAAAAACAAAAAAAAAGCAAT
Co_RmpD_Sall	Reverse primer for the generation of CoCi fragment for <i>rmpD</i> deletion	aaaaa gtcgac AATAAATACTTTTCGCAAAAAACAAT
RmpAF_Kp_qPCR	RT qPCR forward primer for <i>rmpA</i> gene	ACGTCAAGCCACATCCATTG
RmpAR_Kp_qPCR	RT qPCR reverse primer for <i>rmpA</i> gene	ATTCAGGGAAATGGGGAGGG
RmpCF_Kp_qPCR	RT qPCR forward primer for <i>rmpC</i> gene	GGCAAGTCCTGGGTTTACAT
RmpCR_Kp_qPCR	RT qPCR reverse primer for <i>rmpC</i> gene	TGGCCAGCGTCTATTTCAAC
iucF_Kp_qPCR	RT qPCR forward primer for <i>iuc</i> gene	CGCCGCAGTTACTCAATGAA
iucR_Kp_qPCR	RT qPCR reverse primer for <i>iuc</i> gene	CAGTATAGCGAGCGGGAAGA
wcaJF_Kp_qPCR	RT qPCR forward primer for <i>wcaJ</i> gene	TCCTTAGACGAACTTCCGCA
wcaJR_Kp_qPCR	RT qPCR reverse primer for <i>wcaJ</i> gene	ATCTGTTTCACCACGCCAAC
wziF_Kp_qPCR	RT qPCR forward primer for <i>wzi</i> gene	ATCCATCTGAGCCTGTCGAC

wziR_Kp_qPCR	RT qPCR reverse primer for <i>wzi</i> gene	GTTGAAGTGTAGCCGGTGAC
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Table S1. Primers used for this study

Cell	Description	Medium	Seeding density (/cm ²)	Culturing
C3A	Human hepatocytes	Modified Essential Medium, Eagle (MEME +, [Sigma-Aldrich, – Reference: M0268]), containing 10% FCS but no antibiotic	30,000, in MEME+	Media was changed every 2 to 3 days and cells were grown until 80% confluent (usually reached after 5 to 7 days). Only cells below passage 20 were used.
HUVE CS	Human endothelial cells, derived from umbilical cord vein	EGM (Endothelial Cell Growth Medium)-2 (Lonza – Reference: CC-3162), with the addition of EGM BulletKit™ (Lonza – Reference: CC-3124).	25,000, in EGM-2	Media was changed every 2 to 3 days and cells were grown until 80% confluence (usually reached after 7 to 10 days). Only cells below passage 20 were used.
16HBE	Human bronchial	DMEM (Dulbecco's Modified Eagle's Medium, Thermo	120,000, in DMEM	Media was changed every 2 to 3 days and cells were grown until

	epithelial cells	Fisher – Reference: D6046) supplemented with 10% FCS		80% confluence (usually reached after 7 to 10 days). Only cells below passage 20 were used.
RAW 264.7 cells	Murine macrophages	DMEM (Thermo Fisher – Reference: D6046) supplemented with 10% FCS	30,000, in DMEM	Media was changed every 2 to 3 days and cells were grown until 80% confluence (usually reached after 7 to 10 days). Only cells below passage 20 were used.

Table S2. Parameters of cell lines seeding and culture