UNIVERSITY OF TARTU
Faculty of Science and Technology
Institute of Technology

Turgay Hasanov

"Physiological heterogeneity of uropathogenic *Escherichia coli* in bladder epithelial cell infection model"

Master's Thesis (30 EAP)

Curriculum Bioengineering

Supervisor(s):
researcher, Ph.D. Marta Putrins
researcher, MSc Ivana Kerkez
Prof. Dr.Tanel Tenson

Tartu 2023

“Physiological heterogeneity of uropathogenic *Escherichia coli* in bladder epithelial cell infection model”

Uropathogenic *Escherichia coli* (UPEC), which invades host cells, is the primary agent of urinary tract infections. Antibiotics are commonly used to treat UTIs. During the long treatment period, bacteria are getting resistant to antibiotics. To increase understanding of antibiotic treatment efficiency of urinary tract infections an *in vitro* model of intracellular and extracellular infections was needed. Within these, an infection model with HTB-9 human bladder cells and UPEC CFT073 strain has been established. Specific experiments were designed to compare the different conditions to see what the ideal way would be to get the infection to a level where we can use the model to follow infection and antibiotic treatment. To test the viability of the cell, we have compared the CFU results with flow cytometry-based cell counting. The data suggests that bacteria that specifically attach to bladder epithelial cells are targeted by host antimicrobial peptides.

Keywords:

UPEC, urinary tract infection, pathogens, *E. coli*, cell culture infection, bladder, infectious diseases.

CERCS:

B230 Microbiology, bacteriology, virology, mycology, B510 Infections

“Uropatogeense *Escherichia coli* füsioloogiline heterogeensus põie epiteeli nakkusmudelis”

Uropatogeenne *Escherichia coli* (UPEC), kes on võimeline tungima peremeesrakkudesse, on üks peamisi kuseteede infektsioonide tekitajaid. Uroinfektsioonide raviks kasutatakse tavaliselt antibiootikume. Pika raviperioodi jooksul muutuvad bakterid antibiootikumide suhtes resistentseks. Kuseteede infektsioonide antibiootikumravi efektiivsuse mõistmiseks oli vaja rakusiseste ja rakuväliste infektsioonide *in vitro* mudelit. Selle väitekirja raames on loodud nakatumise mudel HTB-9 inimese põierakkude ja UPEC CFT073 tüvega. Konkreetsed katsed kavandati erinevate tingimuste võrdlemiseks, et infektsiooni tase, mis võimaldaks kasutada mudelit infektsiooni ja antibiootikumravi jälgimiseks. Bakterite elumuse testimiseks oleme võrrelnud CFU tulemusi voolutsütomeetria-põhise rakkude loendamisega. Andmed viitavad sellele, et bakterid, mis spetsiifiliselt kinnituvad põie epiteelirakkudele, on peremeesorganismi antimikroobsete peptiidide sihtmärgiks.

Võtmesõnad:

UPEC, kuseteede infektsioon, patogeenid, *E. coli,* akukultuuri infectioon, põis, nakkushaigused.

CERCS:

B230 Mikrobioloogia,bakterioloogia, viroloogia, mükoloogia, B510 Nakkushaigused.

Table of Contents

TERMS, ABBREVIATIONS AND NOTATIONS 6

INTRODUCTION 7

1. LITERATURE REVIEW 8

1. Antibiotics 8

1.1 Mechanisms of Action 8

1.1.1 Alteration of Cell Membrane 8

1.1.2 Inhibition of Cell Wall Synthesis 8

1.1.3 Inhibition of Protein Synthesis 9

1.1.4 Inhibition of Nucleic Acid Synthesis 10

1.2 Killing by Antibiotics 11

1.2.1 Bactericidal and Bacteriostatic 11

1.2.2 Antimicrobial Resistance and Tolerance 12

1.2.3 Antimicrobial Persisters 13

1.3 Urinary Tract Infections (UTI) 13

1.3.1 Importance of Virulence Factors for Uropathogenesis 14

1.3.2 Pathogenesis of UTI 16

1.4. Antimicrobial peptides and other host defense mechanisms. 17

2. THE AIMS OF THE THESIS 19

3. EXPERIMENTAL PART (Materials and Methods) 20

3.1. Materials and Methods 20

3.1.1. DMSO stock preparation, bacterial strain, and Bacterial strain. 20

3.1.2. HTB-9 and J774 cell stock-making and splitting cells 20

3.1.3. Testing Antimicrobial peptides In Growth Media 21

3.1.4. Low CFU testing antimicrobial peptides In Growth Media 22

3.1.5. Infection of HTB-9 Bladder Epithelial Cells 23

3.1.6. Flow Cytometry 24

4.1 RESULTS 25

4.1.1. Establishing of in vitro intracellular model of bladder epithelial cell infection by UPEC 25

4.1.2. Bacterial heterogeneity analysis with flow cytometry. 28

4.1.3 Comparison of CFU/ml and FACS events/ml. 30

4.1.4. Infection model without addition of gentamicin to investigate viability of bacteria attached to the host cells. 32

4.1.5. Comparison of conditioned media of two cell lines HTB-9 and J774 to test the presence of antimicrobial peptides. 35

DISCUSSION 37

SUMMARY 39

Acknowledgements 40

REFERENCES 41

NON-EXCLUSIVE LICENCE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC 47

TERMS, ABBREVIATIONS AND NOTATIONS

AMP - Antimicrobial peptides

AMR - Antimicrobial Resistance

CFU - Colony Forming Units

DMSO - Dimethyl sulfoxide

E.coli - Escherichia coli

FACS - Fluorescence-activated cell sorting

GFP - Green Fluorescent Protein

MBC - Minimum Bactericidal Concentrations

MOI - Multiplicity Of Infection

m-RNA - Messenger RNA

OM – Outer Membrane

PBP – Penicillin-binding proteins

PI - Propidium Iodide

PBS - Phosphate Buffered Saline

rRNA - Ribosomal RNA

SBT - Serum Bactericidal Titer

t-RNA - Transfer RNA

UTI - Urinary Tract Infection

UPEC - Uropathogenic Escherichia coli

INTRODUCTION

Urinary Tract Infections (UTIs) are primarily caused by intracellular bacteria, specifically gram-negative bacteria, such as *Escherichia coli*. UTIs are infections that occur in the urinary system, which includes the kidney, bladder, ureters, and urethra. UTIs are caused by Uropathogenic *Escherichia coli* (UPEC) that invades host cells. While UPEC is the most common cause of UTIs, other pathogens such as *Klebsiella pneumoniae, Proteus mirabilis*, and *Enterococcus faecalis*, among others, are involved. Antibiotics are widely used in the treatment of disease, but they get resistant to the antibiotic when bacteria evolve. Treating UTIs with the correct prescription is important, otherwise, it might lead to antibiotic resistance. If it is left untreated, symptoms can be severe and can lead to complications like sepsis, recurrent infections, and kidney damage(Schmiemann et al., 2010).

Recurrent infection occurs mainly if a person has experienced multiple episodes of UTIs over a period of time. Recurrent UTIs might be frustrating and need to be analyzed for further evaluation and management. The causes of recurrent UTIs are reinfection, residual infections, structural abnormalities, and a weakened immune system. Reinfection is the most common cause, where a new bacterial strain leads to a subsequent UTI after the treatment of the infection successfully from the previous one. Repeated and chronic UTIs happen due to the occurrence of multiple UTIs over a period of time. It can even occur due to the persistence of UTIs despite the treatment. By using the correct treatment, it is possible to resolve the UTIs, but some people are experiencing recurrent or chronic infections, which are challenging to manage.

The aim of this study was to establish an *in vitro* model of infection of bladder epithelial cells with UPEC. To study antimicrobial activity of host cells we used human bladder cell line (HTB-9) and macrophage (J774) cell line. We were able to get more knowledge about pathogenicity and knowledge about the localization of the bacteria in mammalian cells. Also, another aim was to study different bacterial subpopulations by looking at stress response, and dormancy.

1. LITERATURE REVIEW

1. Antibiotics

Antibiotics are antimicrobial substances that fight against bacteria in humans and animals. Antibiotics can kill the bacteria or slow down the multiplication and growth of them. Antibiotics can be classified based on their chemical structures. For example: penicillins, cephalosporins, macrolides, tetracyclines, and quinolones. After the discovery of antibiotics, it was thought that bacterial infections were under control and prevented. Nevertheless, infections continued and started to develop all over the world. The main reason is that resistant infections appeared. Antibiotics can be classified based on the spectrum of activity. Antibiotics can be divided into two categories: broad-spectrum antibiotics and narrow-spectrum antibiotics. Broad-spectrum antibiotics are effective for a wide range of bacteria, while narrow-spectrum antibiotics are effective for a limited range of bacteria.

1.1 Mechanisms of Action

1.1.1 Alteration of Cell Membrane

A cell membrane is made up of two layers of phospholipid molecules. Two layers of phospholipids are arranged with their hydrophobic tails facing inwards, while their hydrophilic heads are turned outwards, and these are the barriers that separate the cell from inside and from outside.

Alteration of the cell membrane can occur in multiple ways, for example, modification of proteins and changes in the composition of lipids, which can affect the fluidity of the membrane and alters the permeability of molecules(Zalba & ten Hagen, 2017). All these alterations have a big impact on membrane and cellular processes. Antibiotics either destroy or slow down the growth of bacteria. The first type of antibiotics are the ones that target cell membranes. This antibiotic will disrupt the cell membrane function, which means that they disrupt the phospholipid bilayer. (Wei et al., 2021).

1.1.2 Inhibition of Cell Wall Synthesis

The cell wall is a rigid layer, protecting bacteria from osmotic stresses and helping to keep the shape. One of the main target structures of antibiotics that can inhibit cell wall synthesis is the peptidoglycan layer. These antibiotics inhibit cell wall synthesis. β-lactams are bactericidal agents which break into bacterial cell walls and target penicillin-binding proteins (PBP).

Bacitracin, vancomycin, and other glyco-peptides prevent the synthesis of cell walls. Antibiotics disrupt the cell wall integrity and make the bacteria more susceptible to osmotic pressure (Hancock & Fitz-James, 1964).

1.1.3 Inhibition of Protein Synthesis

Ribosomes carry out protein synthesis. Ribosomes translate messenger RNA (mRNA) into protein. Ribosomal subunits are created from proteins and ribosomal RNA (rRNA) and have different S-values. These 30S and 50S subunits are ribonucleoproteins, which assemble into 70S ribosome. Aminoglycoside is a natural antibiotic from actinomycetes. To inhibit protein synthesis aminoglycosides, bind to the A-site on the 16S rRNA of the 30S subunit (Krause et al., 2016).

Gentamicin is an aminoglycoside antibiotic, which consists of (C1, C1a, C2, and C2a) and other components (Deubner et al., 2003). It is active against Gram-negative bacteria and creates a perfect opportunity to treat bacterial infections. Gentamicin and other aminoglycosides bind to 16S rRNA of 30S ribosomal subunit, and mRNA translation is interrupted. As with other aminoglycosides, gentamicin depends on concentration for killing bacteria, which means a higher concentration is associated with a greater amount of antimicrobial killing (Sojo-Dorado & Rodríguez-Baño, 2022). This gives clinicians an advantage in using gentamicin in their workplace, such as Urinary Tract Infections (UTI), soft tissue infections, and meningitis (Beganovic et al., 2018).

Other antibiotics, such as tetracyclines, chlortetracycline, and doxycycline, block transfer RNA (t-RNA) binding to the A-site of the 30S subunit (Grossman, 2016; Nelson & Levy, 2011). Chloramphenicol is an antibiotic that attaches to the peptidyl transferase component of 23S rRNA of 50S ribosomal subunit and blocks peptide elongation by preventing t-RNA binding to the A-site (Wang et al., 2019).

Macrolides are bacteriostatic antibiotics that reversibly bind to 23S rRNA of the 50S ribosomal subunit (Lenz et al., 2021). This happens in the early stage of protein synthesis. The lactone ring binds to the exit tunnel of the ribosome, where proteins are synthesized and exit through the tunnel. As a result, macrolide binds to the exit tunnel; the translation and peptide releases are inhibited. As the process continues, t-RNAs are used, and it makes the translation to be stoped (Kannan & Mankin, 2011). Lincosamides and streptogramin B also act like macrolides, inhibiting peptidyl transferase (Tenson et al., 2003).

1.1.4 Inhibition of Nucleic Acid Synthesis

Nucleic acid synthesis inhibitors are a class of antibiotics that target the important processes involved in the synthesis of nucleic acids, including DNA and RNA. These are the antibiotics inhibiting the enzymes, which are responsible for nucleic acid synthesis and prevent the bacteria from multiplying and reproducing.

Inhibitors of DNA and RNA related processes are quinolones and rifamycins. Quinolones are antibiotics, which inhibit bacterial enzymes DNA gyrase for relaxing supercoiling of DNA and topoisomerase IV. It is active for both gram-positive and negative bacteria (PG et al., 2003). By binding to and inhibiting the enzymes, quinolones are able to prevent the DNA from unwinding, which leads to cell death. Rifamycin is an antibiotic that inhibits RNA polymerase. It is involved in the process of transcription, synthesizing the RNA from DNA. Rifamycins block RNA synthesis by binding to the RNA polymerase enzyme. They are not allowing the bacteria to produce the necessary proteins for survival and growth.

DNA gyrase is essential for the relaxing supercoiling of DNA in bacteria, while topoisomerase IV is needed for bacterial genome segregation during cell division. It consists of two subunits of A and two subunits of B (Bhagavan & Ha, 2011). These subunits affect the resistance to drugs. A subunit binds to 5'-DNA during cleavage reaction by the active site of tyrosine residue and allows a negative supercoil to DNA. The B subunit carries ATPase and TOPRIM domain, which binds to metal ions that is important for enzyme activities (Aldred et al., 2014).

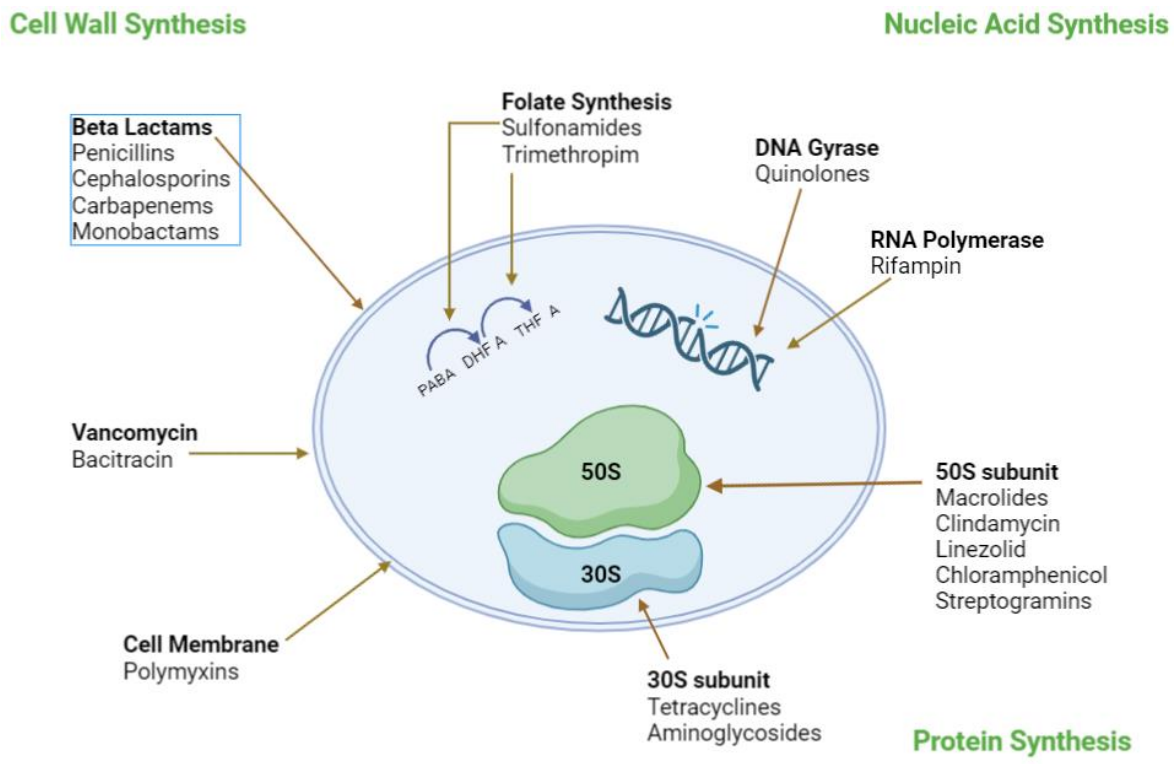


Figure 1. Mechanism of Action of Antibiotics. It consists of Cell wall synthesis, Nucleic acid synthesis, and Protein synthesis.

1.2 Killing by Antibiotics

1.2.1 Bactericidal and Bacteriostatic

Antimicrobials can be classified into two categories, whether the agents kill the bacteria (bactericidal) or agents only prevent the growth of bacteria (bacteriostatic).

Bactericidal antimicrobials inhibit the cell wall, protein translation, or bacterial enzymes by killing bacteria. Some antibiotic agents are listed as examples: Penicillin derivatives, monobactams, cephalosporins, and vancomycin (Pandey & Cascella, 2022). Several techniques have been used to determine the activity of antimicrobial agents by counting the minimum bactericidal concentrations (MBC), serum bactericidal titer (SBT), and time-kill curve (Rhee & Gardiner, 2004).

Bacteriostatic antibiotics are defined as affecting the activity of bacteria without killing them. Bacterial protein production, DNA replication, and protein synthesis pathways are inhibited, and this prevents the bacteria from growing. That is why bacteriostatic antimicrobials need assistance from a stronger immune system. Antimicrobials like macrolides, trimethoprim/ sulfamethoxazole, tetracyclines, linezolid, and clindamycin are bacteriostatic and have been widely used on patients who need treatment of meningitis, endocarditis, osteomyelitis etc.

1.2.2 Antimicrobial Resistance and Tolerance

Antimicrobial resistance (AMR) is when bacteria manage to grow in the presence of antimicrobial agents. AMR happens due to genetic changes in bacteria, fungi, and viruses, resulting in medical treatments do not work properly, and this leads the disease to spread and even cause death (Manfredi, 2006). Nowadays, antibiotics and disinfectants are widely used to prevent the growth of bacteria and kill them.

There are several ways to develop antibiotic resistance. In enzymatic modification, to inactivate antimicrobial agents, acetyl, adenyl, or phosphate groups are added to the antibiotics and do not allow it to bind to target sites (Ramirez & Tolmasky, 2010).

To reduce antibacterial uptake, porins play a key role. It helps the molecule to pass through the porins by diffusion. Porins are considered as the first step or access for antibiotics and are located in the outer membrane (OM). As an example, tetracyclines and beta-lactams pass the OM by porins only (Nikaido, 2011). In gram-negative bacteria, influx reduction or permeability barrier against antibiotics is happening when expression downregulation and porin genes functional deletion occurs. Efflux pumps pump antibiotics out of the cell and keep the low-intracellular concentration low. They are located in the cytoplasmic membranes. Also, efflux pumps are able to pump out unrelated antibiotics before they reach the destination (Poole, 2005).

Tolerant bacteria can withstand high doses of antibiotics, but they do not grow anymore. Bacteria can survive bacteriostatic antibiotics, so the term “tolerance” can only be applied to bactericidal antibiotics. By remaining dormant, it can protect bacteria from various antibiotics (Westblade et al., 2020). Tolerance can be measured by looking at the time-kill curves of an antibiotic’s concentrations (Boldrin et al., 2020). There have been 2 suggestions about forms of tolerance: Tolerance by slow growth and Tolerance by lag. Tolerance by slow growth can be inherited, which means a strain is growing slowly or non-inherited, which has poor conditions

for growth. An example of tolerance by lag is transferring a bacterial species to a new place filled with fresh nutrients and observing the growth resumption delay that gives tolerance to antibiotics (Brauner et al., 2016a; Levin-Reisman et al., 2019).

1.2.3 Antimicrobial Persisters

When bacteria are exposed to antimicrobial agents, two related phenomena take place, persistence, and tolerance. While both persistence and tolerance involve bacterial survival in the presence of antimicrobial agents, they do differ in characteristics and mechanisms. Persistence refers to survival of few bacteria under high exposure to antimicrobial agents, even though the main fraction of the population is killed. Persistent bacteria are phenotypically distinct and enter a dormant state, which exhibits reduced metabolic activity and slowed cellular processes. Persistent bacteria survive the initial antibiotic treatment and later lead to the recurrence of infection after the treatment is stopped (Brauner et al., 2016b). Tolerance refers to the killing of whole population being slower than for wild type. Tolerant bacteria are generally a result of genetic changes that occur during the protection against antimicrobial agents. Also, tolerant bacteria keep active growth and reproduction, unlike the persisters in a dormant state. Tolerance is a heritable trait resulting from genetic or phenotypic changes, allowing bacteria to continue and survive in the presence of agents (Balaban et al., 2019).

Antibiotic persisters are a subpopulation of antibiotic-tolerant bacterial cells, which are growing slower and can continue to grow even after responding to stress. Persistence is non-heritable (Lewis, 2010). If we compare surviving bacteria in time, we will see that there are 2 types: one part is killed, and one small part of the subpopulation persists, which is also called the bimodal or biphasic killing curve (Balaban et al., 2004).

1.3 Urinary Tract Infections (UTI)

Urinary Tract Infections (UTI) are common bacterial infections caused by *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterococcus faecalis,* and *Staphylococcus saprophyticus. UTI causes significant sequelae,* such as renal damage in children, pre-term birth, and pyelonephritis with sepsis in elderly men and women. Also, some complications like high-level antibiotic resistance happen when a person often and continuously uses antimicrobials (Kapoor et al., 2017).

UTI is divided into 2 segments: complicated and uncomplicated. An uncomplicated UTI occurs when an infection is in the lower (Cystisis) and upper (pyelonephritis) urinary tract. Cystitis mainly occurs in women with vaginal infection, obesity, and diabetes. A complicated UTI occurs when an infection spreads to the kidneys and causes serious damage.

Uropathogenic *Escherichia coli* (*E.coli*) (UPEC) is the common agent in UTIs. But there are other agents in both complicated and uncomplicated UTIs **Table 1** (Ronald, 2002).

Table 1. UPEC most common pathogens.

Uncomplicated UTI	**Complicated UTI**
Escherichia coli	*Escherichia coli*
Klebsiella pneumoniae	*Proteus mirabilis*
Staphylococcus saprophyticus	*Enterococcus* spp.
Enterococcus faecalis	*K. pneumoniae,*
Proteus mirabilis	*Candida* spp.
Pseudomonas aeruginosa,	*S. aureus*

1.3.1 Importance of Virulence Factors for Uropathogenesis

Adhesins are molecules on the surface of uropathogenic bacteria that allow them to bind to specific receptors on the urinary tract epithelial cells. This attachment is crucial for bacterial colonization and subsequent infection. Examples of adhesins include type 1 and P fimbriae in *Escherichia coli* (JJ et al., 2000; Shenouda et al., 2020)

Bacteria can form biofilms. Biofilms provide protection to bacteria, making them more resistant to host immune responses and antimicrobial agents. Biofilm formation is associated with persistent and recurrent UTIs. Uropathogenic bacteria may produce toxins that damage the host tissues and enhance their ability to establish infection. For example, hemolysins are pore-forming toxins that can lyse host red blood cells. Other toxins, such as cytotoxic necrotizing

factor (CNF) and alpha hemolysin, contribute to tissue damage and inflammation (Lüthje & Brauner, 2014).

Iron is an essential nutrient for bacterial growth, and uropathogens have developed specific mechanisms to acquire iron from the host. Bacteria produce siderophores, which are small molecules that bind iron and scavenge it from the host. Uropathogenic bacteria can also utilize iron from host proteins and heme sources.

Some uropathogens produce capsules, which are protective layers outside the bacterial cell wall. Capsules help bacteria evade host immune responses by impairing phagocytosis and complement-mediated killing. *Klebsiella pneumonia*, for example, is known for its prominent capsule that contributes to its virulence. The emergence of antibiotic-resistant uropathogens has become a significant concern. Bacteria can acquire resistance genes, either through mutation or horizontal gene transfer, which allows them to survive and persist in the presence of antibiotics commonly used for UTI treatment (Pakbin et al., 2021).

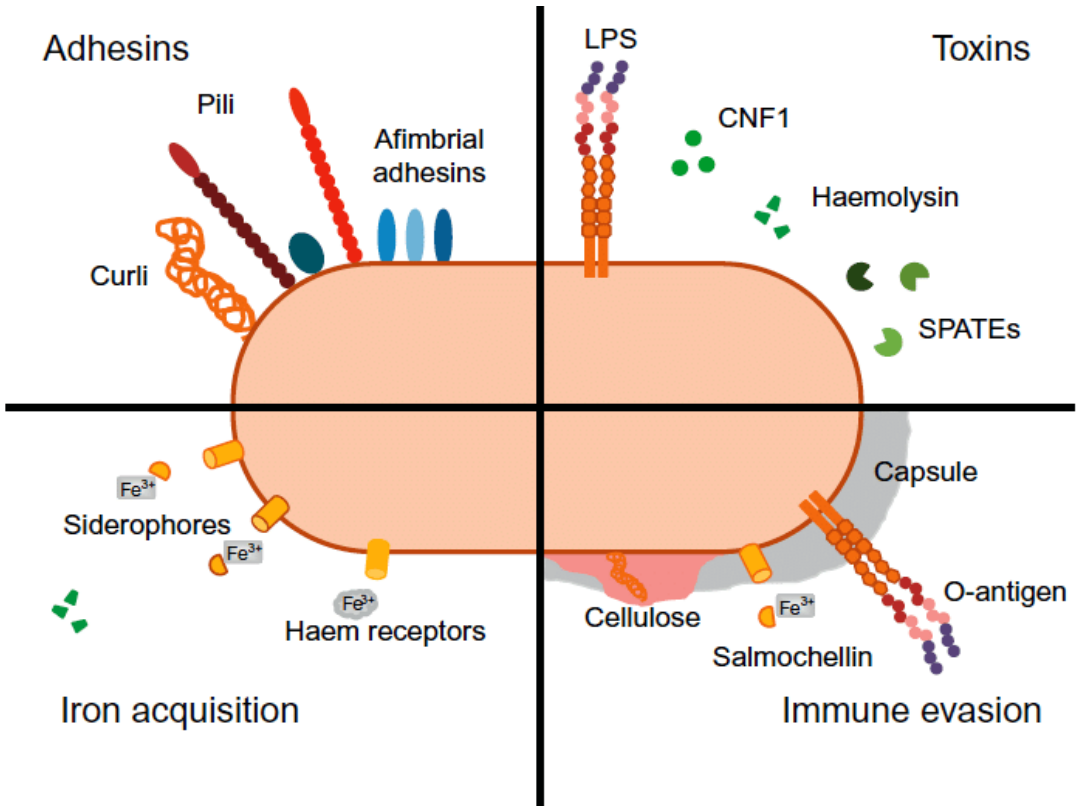


Figure 2. Virulence factors of uropathogenic *E.coli*. Uropathogenic bacteria possess several virulence factors that contribute to their ability to cause urinary tract infections (UTIs) (Lüthje & Brauner, 2014).

1.3.2 Pathogenesis of UTI

The process of bacterial infection involves several stages, including colonization, invasion, internalization, and the intracellular destiny of bacteria. The initial step of infection is the colonization of the host tissues by bacteria. This typically occurs when bacteria adhere to and establish themselves on the surface of host cells or tissues. Adhesion is facilitated by specific bacterial adhesins that recognize and bind to host receptors. Invasion involves the disruption of cellular junctions, induction of host cell signaling pathways, and manipulation of host cellular processes. Bacteria employ various mechanisms for invasion, including the production of bacterial enzymes (e.g., proteases) that degrade host tissues, the induction of host cell endocytosis, or the exploitation of host cell phagocytic pathways (Ageorges et al., 2020).

Internalization occurs when bacteria are engulfed by host cells, such as phagocytes or non-phagocytic cells. Phagocytic cells, including macrophages and neutrophils, engulf bacteria through phagocytosis, forming a phagosome that encloses the bacteria. Non-phagocytic cells, such as epithelial cells, can also internalize bacteria through receptor-mediated endocytosis or other mechanisms.

Once internalized, bacteria encounter the host cell's intracellular environment. The intracellular destiny of bacteria can vary depending on the specific host-pathogen interaction and the bacterial species involved. Bacteria may replicate and survive within host cells, establishing an intracellular niche. This can occur in specialized compartments like the phagosome (e.g., Mycobacterium tuberculosis) or by escaping from the phagosome into the host cell cytoplasm (e.g., Listeria monocytogenes). Some bacteria can manipulate host cell processes to create a favorable environment for their survival and replication. They may inhibit phagosome-lysosome fusion and interfere with host cell signaling pathways. Other bacteria can undergo cell-to-cell spread, exiting the initially infected cell, and invading neighboring cells. This process allows the bacteria to evade the immune system and establish a systemic infection (Belotserkovsky & Sansonetti, 2018; Prado-Silva et al., 2015).

It's important to note that the intracellular destiny of bacteria can vary widely, and different pathogens have evolved distinct strategies to exploit host cells. The ability of bacteria to invade and survive within host cells plays a crucial role in the pathogenesis of various infections, influencing disease severity, immune responses, and treatment strategies.

Uncomplicated UTI starts from the gut, uropathogenic starts contaminating the periurethral area, the urethra begins colonized, and bacteria move to the bladder by pili and flagella,

occupying the bladder (Kim et al., 2018). Neutrophil infiltration starts to clear extracellular bacteria. Some bacteria occupy the immune host cell and destroy the immune system, and some change the morphology, which results in neutrophil resistance. In the next step, biofilm is formed, epithelial damage occurs by toxins and proteases, and released nutrients are needed for bacteria to survive and invade the kidney. As a last step, bacteria keep producing toxins, damage the cells, and make a colony. Complicated UTIs have the same steps as uncomplicated UTIs, but in order to cause infection, they need to bind to the catheter. Fibrinogen-binding proteins make a suitable environment for uropathogenic (Schmiemann et al., 2010). Infection provokes neutrophils, and after the attachment to the catheters, the multiplication of bacteria begins, and biofilms are formed. This is a serious infection that needs to be treated, either wise, it may cross to tubular epithelial barrier and mix with blood (Hunstad & Justice, 2010).

1.4. Antimicrobial peptides and other host defense mechanisms.

Antimicrobial peptides (AMPs) are small proteins that are produced by many living organisms as a defense mechanism against bacteria, viruses, fungi, and other microorganisms. AMPs have been investigated as potential antimicrobial agents for the treatment of infectious diseases. Also, they have been used in other applications, such as cancer therapy and healing of the wound. Disrupting the integrity of the microbial cell membrane by AMPs, it leads to leaks and eventually leads to cell death. This mechanism differs from traditional antibiotics, which often target specific metabolic pathways in bacteria (Abushaheen et al., 2020; Chromek et al., 2006; Kai-Larsen et al., 2010; Mohanty et al., 2022).

One of the primary advantages of AMPs is that they are less likely to induce resistance in microorganisms. This is because AMPs target the membrane of microorganisms, which is a difficult target for microorganisms to modify without compromising their own function. In contrast, traditional antibiotics target specific metabolic pathways, which are more easily altered by microorganisms, leading to the development of antibiotic resistance (Tenover, 2006).

There are other defence mechanisms that are performed by phagocytosis. Phagocytes, including neutrophils, macrophages, dendritic cells, and crucial defense mechanisms performed by immune cells. These cells are able to destroy pathogens, including bacteria, and fuse with lysosomes to form phagolysosomes. Inside the phagolysosomes, pathogens have been put through to the antimicrobial agents and other enzymes, and antimicrobial peptides killed and degraded the pathogens.

Inflammation has been triggered by the host to fight and eliminate the pathogens. It involves immune cells in the infection site and releases inflammatory mediators, such as cytokines and chemokines. Inflammation escalates the microbial activity of immune cells, and as a result, it repairs the tissue (Günther & Seyfert, 2018).

Cellular and Humoral Immunity involves B cells and T cells, which play an important role in eliminating pathogens. B cells produce antibodies to bind to the specific antigens on pathogens by promoting their neutralization and destruction by other immune cells. While T cells are able to directly kill the cells that are infected and can release cytokines, which enhances immune response (Cano & Lopera, 2013).

2. THE AIMS OF THE THESIS

- to establish an *in vitro* model of infection of bladder epithelial cells with UPEC and test various parameters that affect the intracellular infection rate.

- to study different bacterial subpopulations by looking at their stress response, and viability in the developed model.

- To test the hypothesis that bladder epithelial cell produces high quantities of antimicrobial peptides that kill the bacteria attached to the host cell surface.

3. EXPERIMENTAL PART (Materials and Methods)

3.1. Materials and Methods

3.1.1. DMSO stock preparation, bacterial strain, and Bacterial strain.

CFT073 *E.coli* strain was isolated from the urine and blood of a woman with pyelonephritis. It has been transformed withpSC101 plasmid. For the preparation of bacterial dimethyl sulfoxide (DMSO) stocks, over-night culture was diluted in 1:100 Fresh lysogeny broth (Lennox LB; Difco Laboratories) medium. It has grown to an exponential phase in aerobic conditions. DMSO has been added to the final concentration of 8% at an optical density at 600 nm (Od_{600}) of 0,8. Directly the culture was frozen in 120 µl aliquots at -80°C. In our study *E.coli* strain CFT073, was transformed with pSC101 plasmid (Aedla, 2020).

3.1.2. HTB-9 and J774 cell stock-making and splitting cells

HTB-9 cells were derived from bladder carcinoma, and J774 macrophage cells were derived from reticulosarcoma, respectively (R Snyderman, 1977; S Hayashi 1, 1987).
For splitting the HTB-9 and J774 cells, prewarmed 1x Phosphate Buffered Saline (PBS), RPMI (ThermoFisher) medium (including glucose, pyruvate, and hepes in it), and trypsin (CORNING) in the water bath at 37°C for 15 minutes. When the cell confluency reached 85-90%, it was ready for the new passage.

Aspirated the medium above the cells and washed them with 5 ml PBS. Added 5 ml of Trypsin into a T75 flask (ThermoFisher) and put the flask at 37°C incubator for 10-15 minutes to allow trypsin to act. Later, the cells were gently pipetted from the flask to detach and collected into a new 50 ml falcon tube. 10 ml of fresh RPMI media (prewarmed) was added, and the final volume was 15 ml. At this step, took 10 µl from the cell suspension and mixed it with 10 µl of trypan blue stain (ThermoFisher) for cell counting. Calculated the amount needed to make a new passage by Thermo Scientific cell counter and centrifuged the final volume at 120 RCF for 5 minutes.

To make the stocks, added 5 ml RPMI media and 5 ml of FBS into new falcon tubes. Resuspended the cells after the centrifugation. Made calculations for the media needed to be used to concentrate. If the number of cells were above $2*10^6$ cryotubes were prepared and filled. Each tube contains 900 µl of cells and 100 µl DMSO with an 8% final concentration. The cryotubes are stored in liquid nitrogen for an extended period of time.

3.1.3. Testing Antimicrobial peptides In Growth Media

On the first day, seeded eukaryotic cells (HTB-9 an,d J774) on 12-well plates in RPMI medium. Suspension of the cells is prepared, approximately 100 000 cells/ml. Seeding is done at 3 different concentrations with 2 parallels.

- 2ml cells suspension (final number of cells per well 200 000)
- 1ml cells suspension + 1ml media (final number of cells per well 100 000)
- 0.5ml cells suspension + 1.5ml media (final number of cells per well 50 000)

The second day took out the bacteria (CFT073 with GFP-SOS-Scarlet plasmid) and streaked it on to LB plate. Put it to 37°C for 18 hours.
On the third day, cells were grown for 2 days and started to collect supernatant from the 12-well plate. Collected the bacteria with the plastic loop from the agar plate, which was grown for 18 hours, and mixed it with 1xPBS in an Eppendorf tube. Gently pipetted and vortexed for 1 minute at maximum speed.
Measured the OD. Took new cuvettes and inserted 900 µl of 1xPBS and 100 µl of bacterial suspension (approximately 10^9 cells/ml). Made dilutions from 10^9 to 10^5 cells/ml.
Took 10^5 diluted suspension cells and mixed them with supernatant. As indicated in the (**Table 2**), eukaryotic cells are seeded at 3 different time points (Time 0h, 1h, and 2h) with 2 parallels to the 12-well plate. Every 1 hour added bacteria to the corresponding wells by making dilution of 10, 100, and 1000 times.

Table 2. Seeding of Eukaryotic cells at 3 different time points with 2 parallels.

Exp.	T0 10xdil	T0 100xdil	1h 10xdil	1h 100xdil	2h 10xdil	2h 100xdil	2h 1000xdil

3.1.4. Low CFU testing antimicrobial peptides In Growth Media

Ratio of bacteria and antimicrobial peptides is important - when adding too many bacteria, it is possible that killing is not detected.

Started the experiment when HTB-9 or J774 cells were ready for a new passage. To prepare the bacterial cells, took DMSO stock from -80°C, inoculated 100 µl into 3 ml new RPMI media, and grew it in shaking condition for 3 hours at 37°C.

1 ml of supernatant from the HTB-9 and J774 cells were collected (conditioned media) and left on ice to reduce the degradation of antimicrobial peptides that are not very stable. Measured the OD (approximately 10^8 cells/ml) of the bacteria from the shaker and diluted to 10^5 cells/ml. Took 2 fresh media containing and 4 conditioned media containing Eppendorf tubes and put to 37°C incubator for 15 minutes.

Experiment:

- added 10ul of bacterial culture (10^5 cells/ml) to the tubes with media (final concentration of bacteria is approximately 1000 bacteria/ml), kept in a 37°C incubator after each plating session.

- immediately plated out 100 µl to determine initial CFU count.
- After 30 min, plated out 100 µl to see if the killing of bacteria has already taken place.
- after 1h, plated out again 100 µl to see the killing of bacteria.

3.1.5. Infection of HTB-9 Bladder Epithelial Cells

Bacteria:

CFT073 strain transformed with pSC101-GFP-ΔScarlet (AMP resistant) inoculated from DMSO stock (100xdil) into 10ml of LB (100ml flask). Bacteria were grown statically at 37°C for 24 h. After 24h, 100ul of static culture was inoculated into 10ml of fresh LB media and left for an additional 24h, 37°C, and static conditions.

HTB-9 cells were seeded into 12 well plates in RPMI media 2 days before the infection. To count the HTB-9 cells, mixed 10 µl of supernatant and 10 µl of Trypan blue stain 0.4% (ThermoFisher) dye in an Eppendorf tube and counted them on a ThermoFisher cell counter machine.

Centrifuged 2 ml of overnight culture at 180 RCF for 5 minutes. Pellet was suspended in 2 ml of RPMI media. OD was measured at 5-, 10-, and 20-times dilutions, respectively. Calculated which volume of bacteria is needed to get the appropriate Multiplicity of Infection (MOI). 2ml of RPMI media with 10% FBS-containing bacteria were added into each well (above bladder cells) and centrifuged at 110 RCF for 5 minutes. After centrifugation, put 12 well plates in a CO_2 incubator (Memmert) for 2 hours for the phagocytosis step. The medium was removed, and Gentamicin 100 µl/ml in RPMI with 10% FBS was added to each well for 2 hours to kill all extracellular bacteria. Accumulation of gentamicin into mammalian cells is very low and therefore intracellular bacteria are protected from killing by gentamicin. Washed all wells 2 times with 1xPBS to remove the extracellular bacteria and leftovers of gentamicin.

CFU plating preparation:

Time zero samples were lysed into 1 ml of filtered water, and 50 µl was plated on an LB agar plate for CFU counting. The second plate with infected cells was incubated for an additional 24 hours in the presence of 10xMIC of gentamicin. After 24 hours, well-containing gentamicin was washed 2 times with 1xPBS and lysed in 1 ml of filtered water. 50 µl of samples were plated onto the LB agar plate for CFU determination.

FACS sample preparation:

When cells were lysed in 1ml of water: the sample was first divided into 2 Eppendorf's tubes, 500 µl in each tube.

1. One is used for CFU plating – 500 µl were vortexed and pipetted gently, and 50ul of the sample was directly added to the LB plate and spread with beads and protein determination.

2. Another 500 µl should be centrifuged at 180 RCF for 5min, and the pellet was dissolved in 1ml of 1xPBS. Facs sample is divided once more into 2 Eppendorf tubes.

500ul – no Propidium Iodide (PI) dye.

500 µl with PI dye.

In both tubes, 1XPBS was added to reach the final volume of 1 ml.

3.1.6. Flow Cytometry

Flow cytometry has been used to detect and quantify particles or cell populations. Flow cytometry is measuring and analyzing the cell population by letting them pass singularly through a laser. The particles are analyzed for light scatter and fluorescence characteristics. There are two orientations at a 90° angle (side scatter, SSC) indicating the granularity of the cell and forward direction (forward scatter, FSC) which indicates size of a cell in the light scatter.

Bacteria samples were diluted, and concentrations were adjusted to 10^6 CFU in 1 x PBS. Later, added cell suspension to two Eppendorf tubes (500 µl to each), centrifuged cell suspension at 12000 rpm for 5 minutes. Dissolved pellets in 1 x PBS. Added MM 4-64 dye (Enzo) (Ex=561 nm, Em=695/40nm) to one of the tubes, and the second one we did not add. Final concentration of the added dye tube was 2 µg/ml. Left the tubes in room temperature for 15 minutes and analyzed them with flow cytometer (Attune NxT Flow Cytometer). Ex=488 nm, Em: 530/30 was used for green fluorescence.

4.1 RESULTS

4.1.1. Establishing of in vitro intracellular model of bladder epithelial cell infection by UPEC

To establish UPEC *in vitro* intracellular infection model, we have been working with cancer cell line HTB-9. Our experimental setup is indicated in (Fig. 3). By developing an infection model utilizing a human bladder cell line and fluorescent uropathogenic *Escherichia coli* (UPEC), we obtained valuable insights into bacterial localization and the pathogenesis process. Additionally, this model holds significance in future studies evaluating the effectiveness of both widely prescribed antibiotics for UTI treatment and novel drugs currently undergoing clinical trials.

Figure 3. shows the infection model of the experiment. 2 hours after bacteria and host cells are mixed together, gentamicin was added to the cell culture to kill bacteria that are not internalized. Number of intracellular bacteria was analyzed after 1h of gentamicin treatment (Time 0) or after 24h (Time 24h).

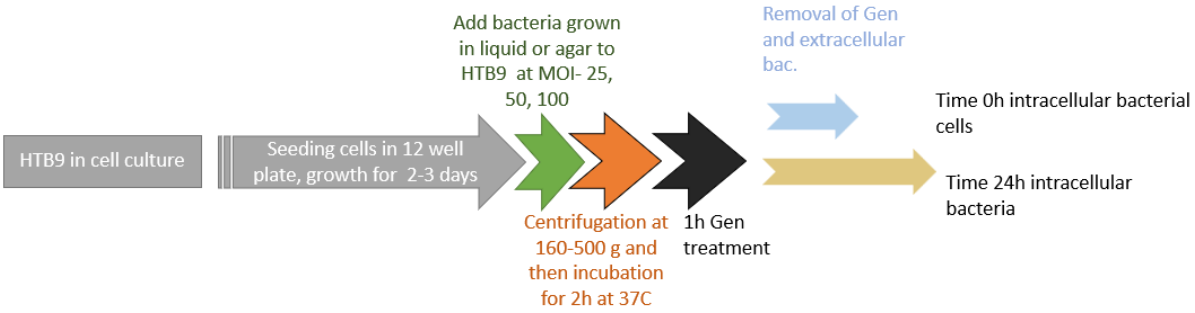


Figure 3. Schematic representation of infection protocol. Bacteria were grown in liquid or on solid agar media. Experiment was performed at three different MOIs (25, 50, and 100). Two different centrifugations speeds (160g and 500g) were used to facilitate contact between bacteria and host cells. Gentamicin (100 mg/liter) was used to kill the extracellular bacteria.

Several experimental details were varied to see how it affects the final intracellular infection rate. First, we have tested bacterial pregrowth conditions – bacteria were grown before infection experiment either on agar plates or in liquid static culture. The ratio between host cells and infecting pathogen might affect how many of the host cells get infected and how big is intracellular load of pathogen. Therefore, UPEC strain CFT073 was used with HTB-9 cells at different ratios. Here the aim was to check the bacterium/host cell ratios (multiplicity of infection, or MOI) (**Fig 4.**). In our first experiment centrifugation to facilitate the contact between bacteria and host cells was performed at 160g and host cells were lysed with H_2O before plating bacteria for CFU determination.

In the experiments, we got approximately 1 mg of host cells protein per well in a 12-well plate that corresponds to approximately 100 000 cells. At timepoint 0h, bacteria growing on agar were showing similar intracellular bacterial loads achieved in all MOI-s, while in liquid media infection was 2 times bigger (**Fig 4**). When we look at the data on timepoint 24 hours, both conditions agar and liquid intracellular bacterial count have started to decrease (**Fig 4**). Comparing the results to another experimental results with macrophage-like cell line (Kerkez et al., 2021) we can see that there is a 1000 times lower intracellular infection, which was expected in our case. When comparing agar and liquid, there is a big difference, and it could be explained by bacterial piliation that would enhance attachment to host cells. It is unknown why the liquid-media statically grown bacteria infect at higher rates. Maybe in the experiment, there was more pili expressed in liquid media condition which led to this difference.

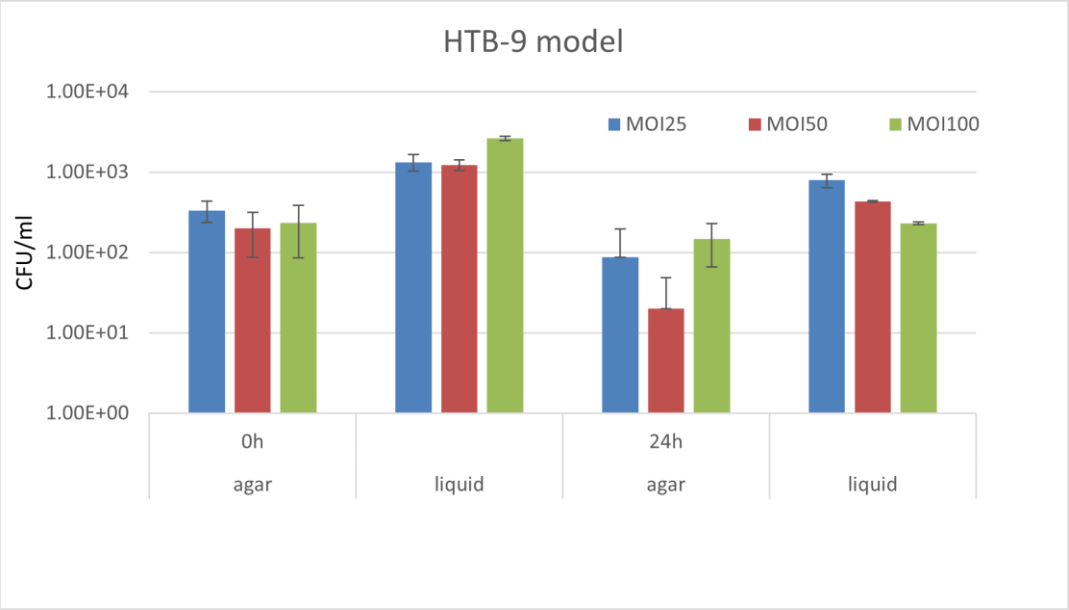


Figure 4. Number of intracellular bacteria at time zero and at time 24 hours with different infection loads, shown as MOI. There is an average of 1-3 parallels from 2 independent experiments.

In the next experiment we modified centrifugation speed in the step where bacteria and host cells are mixed together. The idea is that if bacteria become in strong contact with the host cells it will result also in better uptake of bacteria and therefore also in higher intracellular bacterial numbers.

When we look at the number of intracellular bacteria (**Fig.5**), we can see almost 2 times differences between the MOIs that are growing on agar compared to liquid static, like in previous experiment. At timepoint 0h, bacteria growing on agar were showing stable and similar results in all MOIS, while in liquid, number of intracellular bacteria kept growing as the MOI get higher (**Fig 5**). At timepoint 24h, there was a sudden decrease in both conditions like in previous experiment (**Fig 4 and 5**). The bacterial cells have started to lose their viability and to die. There were 2 centrifugations speeds, 160g and 500g used in the step where bacteria are added to the HTB-9 cell culture. Figure 5 represents that centrifugation has not changed level intracellular bacteria, which means increasing the centrifugation speed, is not affecting too much in conditions were bacteria are pre-grown on agar plates (**Fig 5**). We can assume that already at 160 g the contact between bacterial cells and host cells is achieved. However, when bacteria are pre-grown in liquid culture, we can see that higher centrifugation speed in experimental setup always results in higher intracellular loads of bacteria (**Fig 5**).

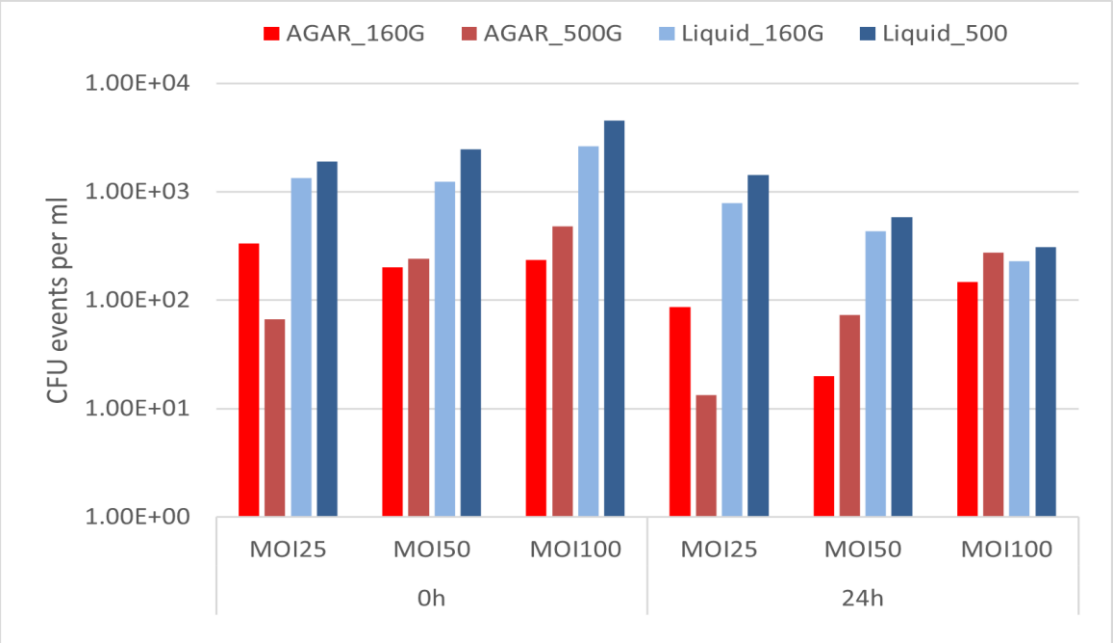


Figure 5. HTB-9 intracellular infection model with *E.coli* CFT073. This graph represents the number of viable intracellular bacteria measured as CFU/ml**.** Samples are taken and analyzed at two different time points: time zero and time twenty-four hours at 3 different infection loads (MOI25, MOI50, and MOI100), shown as the multiplicity of infection (MOI). In addition, two conditions have been tested, agar and liquid static, respectively, and two different centrifugation steps have been implemented, 160g and 500g, for both conditions. There is an average of 1-3 parallels from 2 independent experiments.

4.1.2. Bacterial heterogeneity analysis with flow cytometry.

Analysis of viable intracellular bacterial numbers by CFU determination and total numbers of bacterial cells by flow cytometry (FACS events) can help to compare the different conditions: agar and liquid, to see what the ideal way would be to get the infection to a higher level, where we can use the results and data to follow it and study further. In the developed infection model not only the infection rate is important but also expression of fluorescent reporter-proteins in bacterial cells.

FACS has allowed us not only to count the cells but also to analyze the fluorescence and size of single cells in the population of bacteria. It has let to find out where the bacteria express the fluorescent reporter-proteins that could be in future studies used to detect intracellular bacteria (based on GFP signal) and to analyze their stress response (based on mScarlet signal).

A. LB Agar *B.* LB Liquid-Static

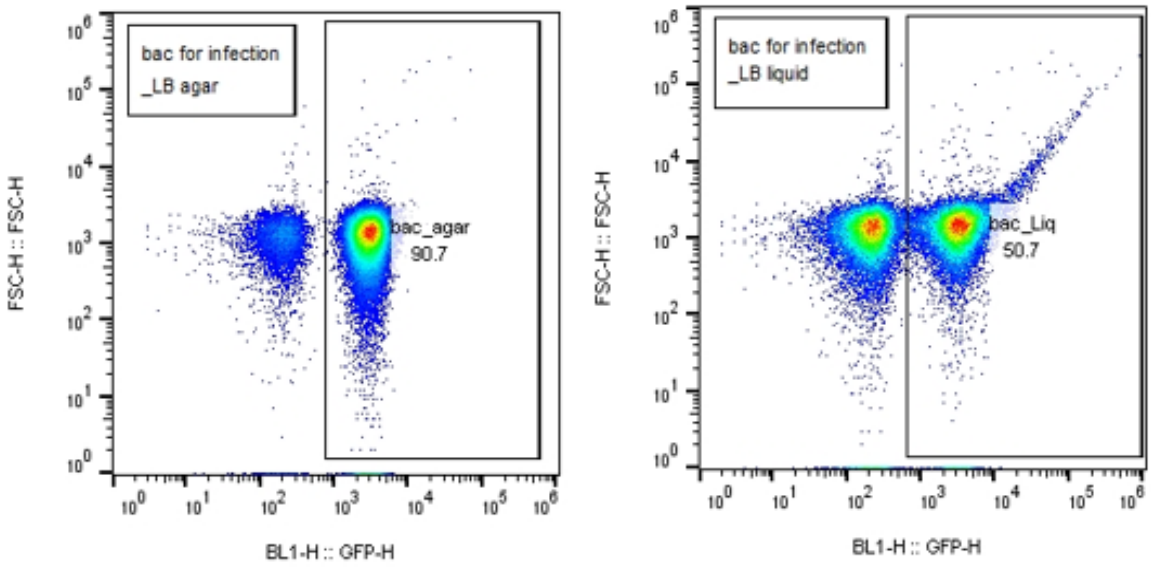


Figure 6 A and B. Analysis of bacterial populations with flow cytometry. A – bacteria are grown on LB agar plates. aB – bacteria are grown in liquid LB. Bacteria are analyzed before the cultures are added to the host cells. Forward scatter height (FSC-H) used to measure the size of the cells. Green fluorescence (BL1-H::GFP-H) is used to measure the relative amount of GFP single cells express.

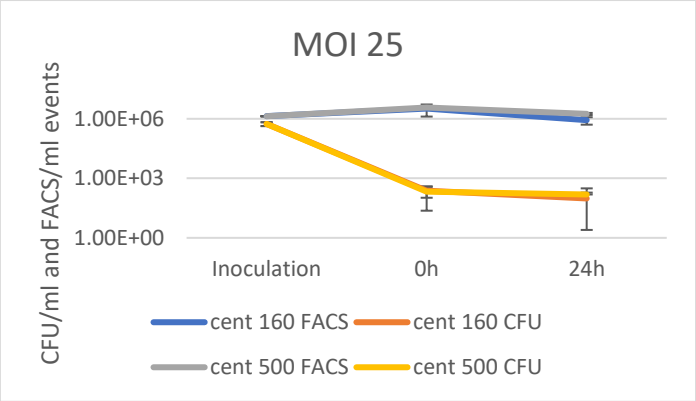
To analyze if all bacteria express equally GFP protein cells were first stained with membrane-binding dye FM4-64. Bacterial populations are gated first based on FM-4-64 signal and then analyzed based on the expression of green fluorescent protein (GFP) **(Fig 6A, B).** For the bacteria that are growing on agar media, the fluorescence reporter GFP was more equally expressed. Comparing LB agar **(6A)** to LB Liquid-Static **(6B)** can clearly indicate that many bacteria are not expressing GFP in Liquid Static and it is difficult to analyze them later. GFP maturation might be the reason. GFP maturation needs oxygen, and in the static culture maybe the ones growing in the bottom are receiving less oxygen. Another reason might be that transcription is not going on, so that protein is not maturating so efficiently.

4.1.3 Comparison of CFU/ml and FACS events/ml.

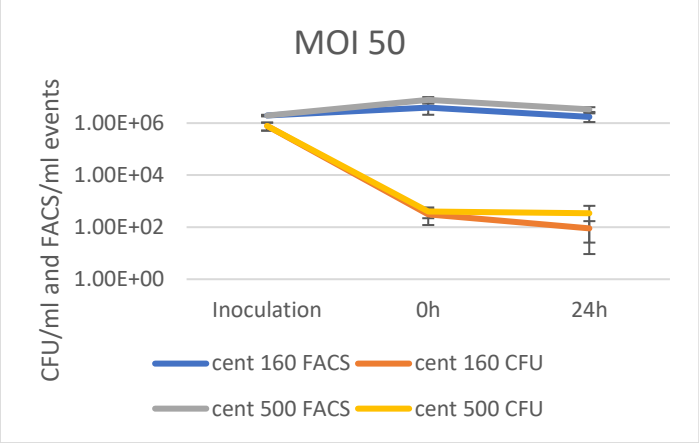
Next, we compared viable bacterial cell numbers determined by CFU plating with total number determined by flow cytometry analysis of green fluorescence bacteria. In Figure 7 below, the CFU and FACS bacterial populations were compared. Experiments have been done in three different MOI conditions and at two different centrifugation speeds.

The graphs show lower CFUs and events in flow cytometry. There is a minor difference between CFU and total number of bacterial cells counted with flow cytometry already in cultures used for infection. Not all bacteria can form colonies. Because during technical steps, such as dilution, some bacteria get lost, or during plating, two bacteria could be very close to each other, we might get one colony, but in flowcytometry we still get double events.

A. MOI 25



B. MOI 50



C. MOI 100

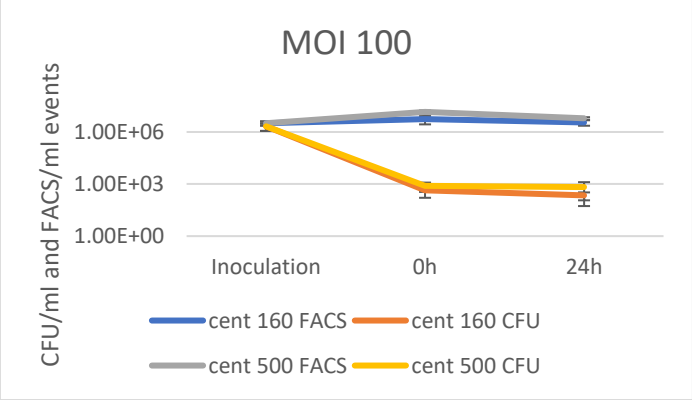


Figure 7. A, B and C represent comparison of CFU and FACS data of bacterial population from three different MOI (25, 50 100) conditions. Centrifugation was done at 160g and 500g. Blue and grey lines show FACS data, while orange and yellow represent CFUs. There is an average of 1-3 parallels from 2 independent experiments.

Basically, the number of bacteria detected by Flow cytometry is not changing during the time, but there is a drastic drop in CFU levels already in the Time 0h because of addition of gentamicin that kills all extracellular bacteria. The aim was to check if increasing the MOI (25, 50, 100) and speed of centrifugation (160g and 500g) would affect the results, it seems it did not. Because killing effect is the same in all conditions in the MOI. CFU goes down, still can see the bacteria in flow cytometry. Next, we wanted to see if there is the killing also before gentamicin was added to the cell culture to test the hypothesis that host cells produce antimicrobial peptides that kill attached bacterial cells.

4.1.4. Infection model without addition of gentamicin to investigate viability of bacteria attached to the host cells.

In the next experiment we compared CFU and flow cytometry measurements of bacterial cells before addition of gentamicin. This experimental scheme of the HTB-9 model of the infection is shown in Figure 8.

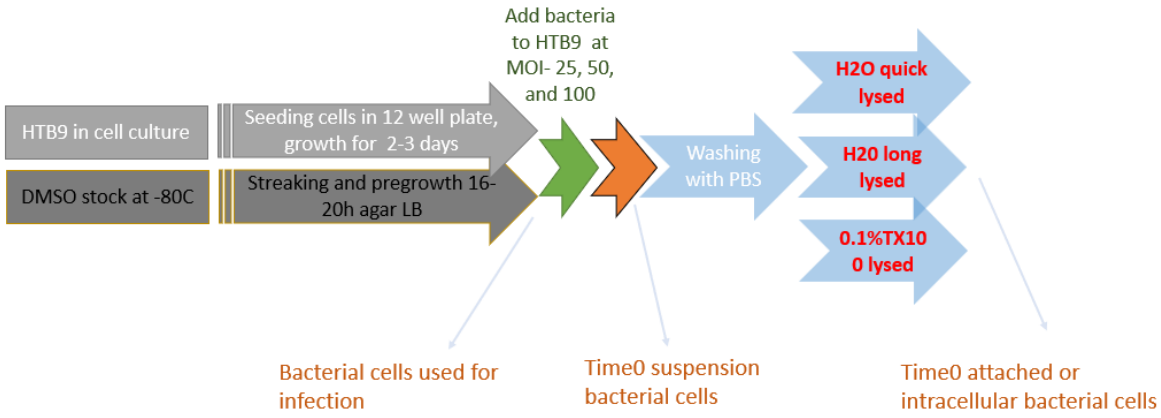
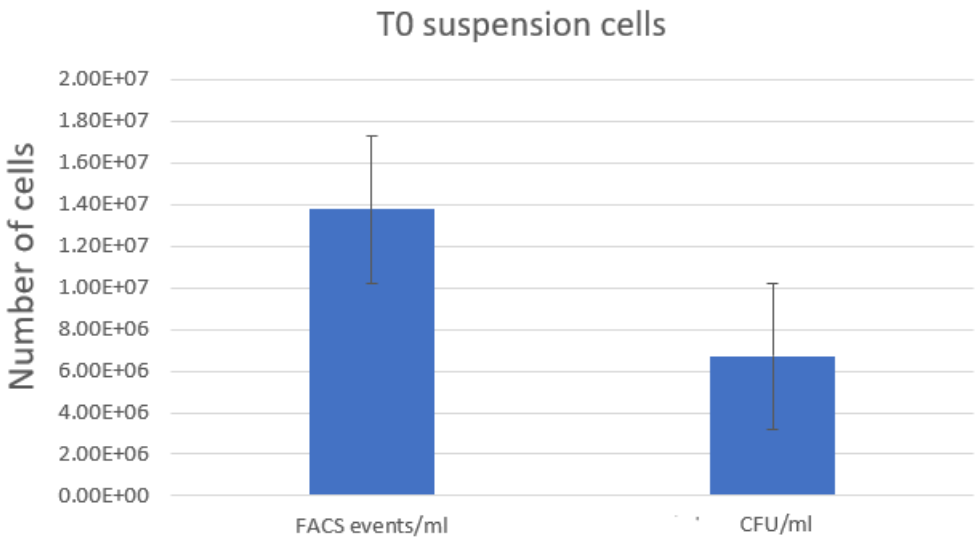


Figure 8. Schematic representation of infection protocol. Three different lysing steps have been used, two of them with water, divided into two parts: quick lysing and long lysing (15 min), and 1 of them with 0.1% times TX100.

We mixed together HTB-9 cells and bacterial cells that were grown on agar media and incubated for 2h. If we check the bacterial cells growing in medium above the HTB-9 cells (**Fig. 9A**) there is little difference between bacteria counts measured by flow cytometry and

CFU. If we look at the bacteria that are in contact with the host cells (**Fig. 9B**) that can be either attached bacteria or intracellular, then the difference between number of bacteria measured either by CFU plating or flow cytometry is already bigger. It means that, even if we do not add any drugs to the experimental system, we still get killing of bacterial cells.

A. Suspension cells



B. Attached + Intracellular

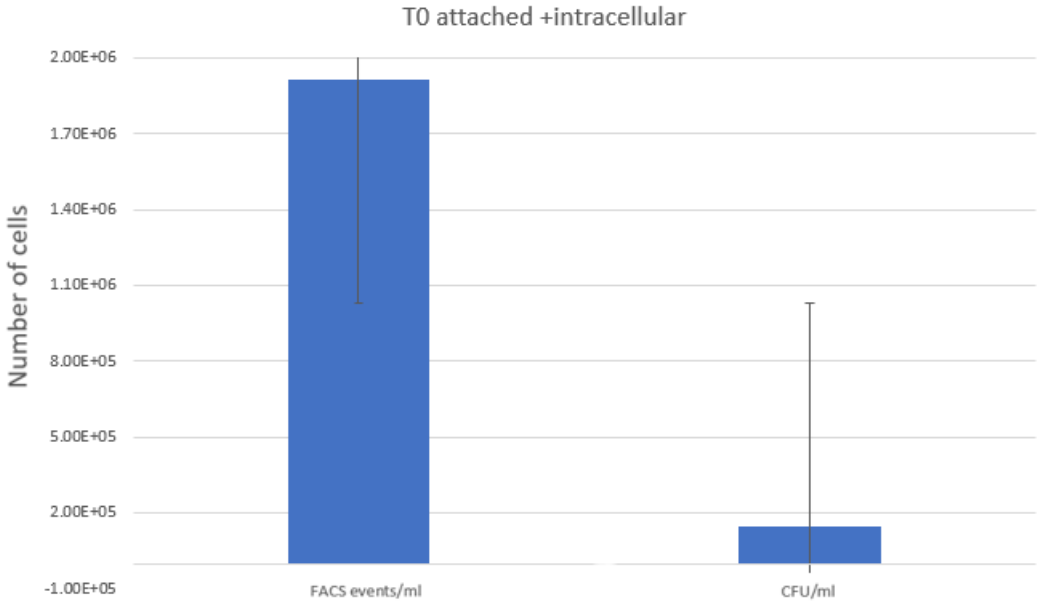


Figure 9 A and B. Represents suspension cells and Time0 attached or intracellular bacteria.

The aim of the next experiment was to check if the viability of the bacteria is influenced by the handling of the samples. While bacterial cells growing in medium above the HTB-9 cells are plated directly, then attached and/or internalized cells are plated only after the step where host cells are lysed. On Figure 10 we can see bacterial viability that is calculated as a percentage of bacteria detected by flow cytometry giving CFU-s. Before, bacteria were added to HTB-9 cells. Approximately 80% of them are viable (giving colonies after plating) (**Fig 10**) After the bacteria being in contact with eukaryotic cells in suspension, 50% of them were viable (**Fig 10**). But when we look at the attached or internalized bacteria, we see more than 90% loss of the viability of bacterial cells. In the attached infection, quick lysing with water had the highest viability, while quick lysing with 0.1%TX100 has the lowest. We can conclude from that experiment that while there were small differences depending on sample handling it does not affect the main result that bacteria are killed by host cells.

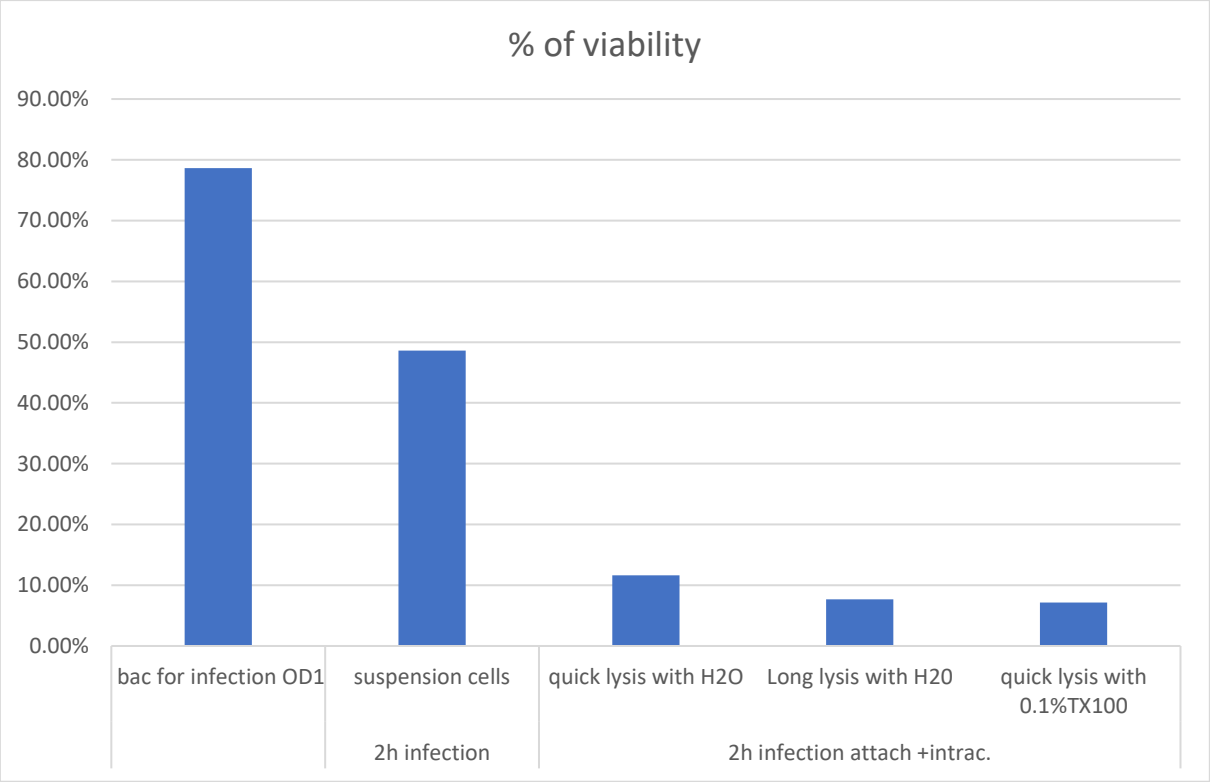


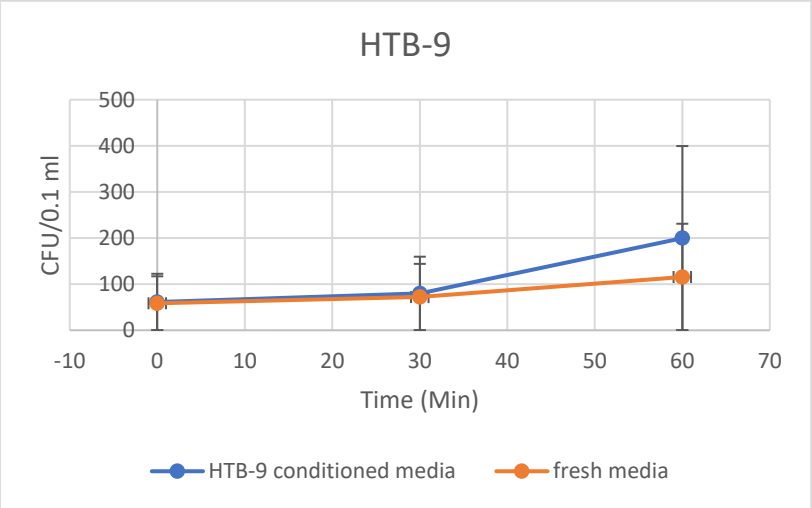
Figure 10. The graph shows the viability of the bacteria in suspension cells, and in attached or intracellular cells.

Discovered that in the infection model, we do not just look at bacteria growing inside or outside, but we have got attachment and this attachment results in antimicrobial activity from the host and this makes the HTB-9 model much more complicated.

4.1.5. Comparison of conditioned media of two cell lines HTB-9 and J774 to test the presence of antimicrobial peptides.

To test the hypothesis that host cells produce any antimicrobial peptide, we decided to compare two cell lines, HTB-9 and J774. Most of the antimicrobial peptides do not diffuse far away from the cell surface. Also, there might not be very high quantities of antimicrobial peptides in the conditioned media, we wanted to see if conditioned media inhibits growth of bacteria more than fresh eukaryotic growth media.

A. HTB-9 cell line



B. J774 cell line

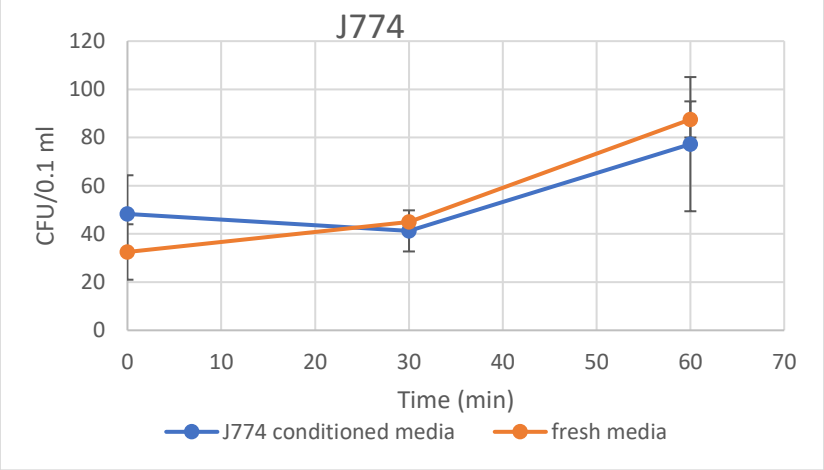


Figure 11. Comparison of HTB-9 (A) and J774 (B) cells respectively. Exposed the conditioned media to bacteria to compare their antimicrobial peptide activity. Blue line indicates conditioned media, while orange line indicates fresh media.

In Figures 11 A, and B, we have exposed bacterial cells to the conditioned media collected from cell culture plates of HTB-9 and J774 cells in comparison with fresh eukaryotic growth medium. We could not detect the antimicrobial activity by just taking the conditioned media (**Fig 11**). The reason might be eukaryotic cells are growing without any expose to bacterial cells in the media and antimicrobial peptides do not get expressed. Then the expression level of antimicrobial peptides is too low to detect its effect on bacterial cells.

Overall, we could not see any killing, by just collecting supernatant, maybe these antimicrobial peptides are not sufficiently induced. When we collected and filter-purified the supernatant from host cells that were exposed to bacteria, and used it as growth media for UPEC, it let us to see some killing (**Fig 12**).

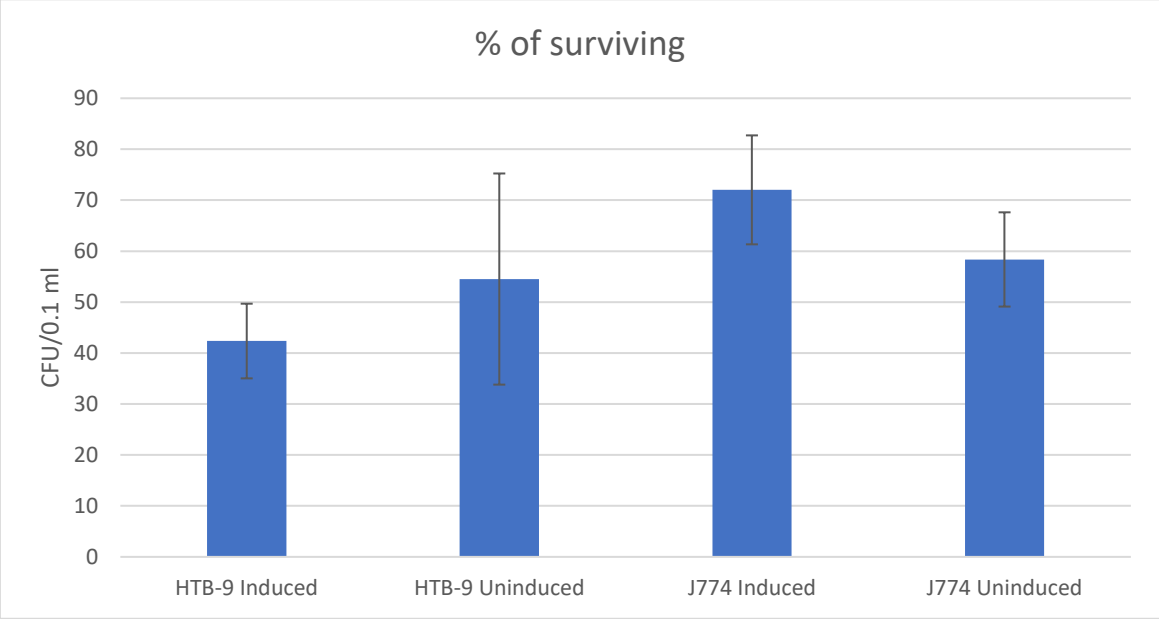


Figure 12. Experiment of induced and uninduced cell lines and bacterial survival in collected supernatants. Average and standard deviation of three parallels is shown.

Exposed both cell lines to bacteria and then collected conditioned media to induce the production of antimicrobial peptides (**Fig 12**). Conditioned growth media collected from HTB-9 after exposure to bacterial cells (HTB-9 induced) has the lowest number of surviving bacteria rate among the others. It is not easy to extract the antimicrobial peptides from the supernatant, because they tend to stay attached to the surface. When we look at the induced data, we can see the highest difference there between HTB-9 and J774.

DISCUSSION

Our aim was to establish an in vitro model of infection of bladder epithelial cells with UPEC. We aimed to have an infection model, which will be used to study antibiotics against intracellular bacteria.

We first tested several experimental conditions in order to achieve high intracellular infection levels. The highest MOI we have tried in the experiments was MOI 100. If we had gone to higher MOIs, it would have been possible that we could have destroyed the bladder cells. During high infection by bacteria, the lysis of eukaryotic cells can occur, and if it is not toxic for bladder cells, we can look forward and try out new experiments.

FACS has allowed us to analyze the fluorescence, but in the results, we noticed depending on growth condition bacteria are not always expressing GFP (**Fig 6B**). There can be several reasons why bacteria may not express a green fluorescent protein or loss of GFP-Encoding Gene. But this plasmid we have used is stable plasmid, which means it is not very likely to disable. Another reason could be protein degradation. Even if the GFP gene is properly inserted and expressed, the stability of the GFP protein can vary. Bacterial proteases or other degradation pathways may target GFP for degradation, resulting in low or undetectable GFP fluorescence (Bragança & Kraut, 2020). Also, regulatory factors might be the reason. Bacteria possess regulatory mechanisms that control gene expression. There might be specific factors or conditions that prevent the activation of GFP gene expressions, such as the absence of necessary inducing signals.

First, we got only a low number of intracellular bacteria into HTB-9 (**Fig 4**). Secondly, discovered that bacteria are dying in the infection model before adding any antibiotics into the cell culture (**Fig 9B**). This means that they are getting actively killed. The finding was based on experiment data where we could count bacteria with flow cytometry but most of them did not form colonies. This has led to the hypothesis that, HTB-9 cells produce high number of antimicrobial peptides that will kill bacterial cells that attach to the surface which is supported also by literature data (Chromek et al., 2006; Mohanty et al., 2022).

HTB-9 cells are a type of human cell line used in research studies to investigate the activity of antimicrobial peptides. Studies have shown that HTB-9 cells can produce several types of antimicrobial peptides, including defensins, cathelicidins, and histatins (De Smet & Contreras, 2005). These peptides have demonstrated activity against various microorganisms, making

them a promising target for developing new antimicrobial agents. Additionally, AMPs have been shown to have immunomodulatory effects, meaning they can help to modulate the immune response. This can be particularly useful in HTB-9 cells, which are involved in the immune response. The immune response primarily involves various immune cells such as T cells, B cells, macrophages, dendritic cells, and others. These cells are responsible for recognizing and responding to foreign antigens, pathogens, and other immune-related processes. (Pahar et al., 2020).

SUMMARY

Antibiotics have an essential role in fighting urinary tract infection. In this study we used HTB-9 bladder epithelial cell line with CFT073 strain to check the heterogeneity of uropathogenic *Escherichia coli* in bladder epithelial cell infection model. We also studied the different bacterial subpopulations by looking at their stress response and viability.

To study the intracellular antibiotic activity in vitro, we did an optimization experiment including different overnight growing bacteria with different conditions – on lysogeny broth (LB) and LB liquid static) at 37°C. Analyzed the data using CFU and FACS.

The aim was to compare the different conditions, agar and liquid, to see the ideal method of getting a higher level of infection. In the experiment bacterial internalization was done at different MOIs at two different time points (0h and 24h) and at timepoint 0h, we got higher MOI results, comparing it to timepoint 24h. This means a mechanism from the host side controls the infection and bacteria starts to lose viability.

Also, to check the viability of the cells, comparison of CFU results and FACS data were done. Noticed that flow cytometry is not changing during the time, while bacterial viability based on CFU counting it is dropping suddenly even before adding antibiotics to the system. This means, bacterial cell is getting killed actively by the host cells which makes that developed model very complex and challenging.

Acknowledgements

This study was performed in Professor Tanel Tenson laboratory at the Institute of Technology.

The completion of this study could not have been possible without my supervisors Ph.D. Marta Putrins, MSc Ivana Kerkez. I would like to special thank them for their patience, support, and positive attitude, for their guidance and encouragement to accomplish this assignment.

I would also like to thank Prof. Dr.Tanel Tenson for providing this condition and giving me a chance to be a part of his lab group. My deepest appreciation goes to the people working in Tenson lab, it was a great pleasure to work with them and appreciate their help, ideas, and thanks for providing such a good learning environment.

REFERENCES

Abushaheen, M. A., Muzaheed, Fatani, A. J., Alosaimi, M., Mansy, W., George, M., Acharya, S., Rathod, S., Divakar, D. D., Jhugroo, C., Vellappally, S., Khan, A. A., Shaik, J., & Jhugroo, P. (2020). Antimicrobial resistance, mechanisms and its clinical significance. *Disease-a-Month : DM*, *66*(6). https://doi.org/10.1016/J.DISAMONTH.2020.100971

Aedla, J. (2020). *Antibiotic detection using bioreporters based on SOS and cold shock responses*. https://dspace.ut.ee/handle/10062/72054

Ageorges, V., Monteiro, R., Leroy, S., Burgess, C. M., Pizza, M., Chaucheyras-Durand, F., & Desvaux, M. (2020). Molecular determinants of surface colonisation in diarrhoeagenic Escherichia coli (DEC): from bacterial adhesion to biofilm formation. *FEMS Microbiology Reviews*, *44*(3), 314–350. https://doi.org/10.1093/FEMSRE/FUAA008

Aldred, K. J., Kerns, R. J., & Osheroff, N. (2014). Mechanism of Quinolone Action and Resistance. *Biochemistry*, *53*(10), 1565. https://doi.org/10.1021/BI5000564

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., Brynildsen, M. P., Bumann, D., Camilli, A., Collins, J. J., Dehio, C., Fortune, S., Ghigo, J. M., Hardt, W. D., Harms, A., Heinemann, M., Hung, D. T., Jenal, U., Levin, B. R., … Zinkernagel, A. (2019). Definitions and guidelines for research on antibiotic persistence. *Nature Reviews. Microbiology*, *17*(7), 441–448. https://doi.org/10.1038/S41579-019-0196-3

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L., & Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science (New York, N.Y.)*, *305*(5690), 1622–1625. https://doi.org/10.1126/SCIENCE.1099390

Beganovic, M., Luther, M. K., Rice, L. B., Arias, C. A., Rybak, M. J., & Laplante, K. L. (2018). A Review of Combination Antimicrobial Therapy for Enterococcus faecalis Bloodstream Infections and Infective Endocarditis. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *67*(2), 303–309. https://doi.org/10.1093/CID/CIY064

Belotserkovsky, I., & Sansonetti, P. J. (2018). Shigella and Enteroinvasive Escherichia Coli. *Current Topics in Microbiology and Immunology*, *416*, 1–26. https://doi.org/10.1007/82_2018_104

Bhagavan, N. V., & Ha, C.-E. (2011). DNA Replication, Repair, and Mutagenesis. *Essentials of Medical Biochemistry*, 287–299. https://doi.org/10.1016/B978-0-12-095461-2.00022-9

Boldrin, F., Provvedi, R., Cioetto Mazzabò, L., Segafreddo, G., & Manganelli, R. (2020). Tolerance and Persistence to Drugs: A Main Challenge in the Fight Against Mycobacterium tuberculosis. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/FMICB.2020.01924

Bragança, C. E., & Kraut, D. A. (2020). Mode of targeting to the proteasome determines GFP fate. *The Journal of Biological Chemistry*, *295*(47), 15892. https://doi.org/10.1074/JBC.RA120.015235

Brauner, A., Fridman, O., Gefen, O., & Balaban, N. Q. (2016a). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nature Reviews. Microbiology*, *14*(5), 320–330. https://doi.org/10.1038/NRMICRO.2016.34

Brauner, A., Fridman, O., Gefen, O., & Balaban, N. Q. (2016b). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nature Reviews. Microbiology*, *14*(5), 320–330. https://doi.org/10.1038/NRMICRO.2016.34

Cano, R. L. E., & Lopera, H. D. E. (2013). *Introduction to T and B lymphocytes*. https://www.ncbi.nlm.nih.gov/books/NBK459471/

Chromek, M., Slamová, Z., Bergman, P., Kovács, L., Podracká, L., Ehrén, I., Hökfelt, T., Gudmundsson, G. H., Gallo, R. L., Agerberth, B., & Brauner, A. (2006). The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection. *Nature Medicine 2006 12:6*, *12*(6), 636–641. https://doi.org/10.1038/nm1407

De Smet, K., & Contreras, R. (2005). Human antimicrobial peptides: defensins, cathelicidins and histatins. *Biotechnology Letters*, *27*(18), 1337–1347. https://doi.org/10.1007/S10529-005-0936-5

Deubner, R., Schollmayer, C., Wienen, F., & Holzgrabe, U. (2003). Assignment of the major and minor components of gentamicin for evaluation of batches. *Magnetic Resonance in Chemistry*, *41*(8), 589–598. https://doi.org/10.1002/MRC.1222

Grossman, T. H. (2016). Tetracycline Antibiotics and Resistance. *Cold Spring Harbor Perspectives in Medicine*, *6*(4). https://doi.org/10.1101/CSHPERSPECT.A025387

Günther, J., & Seyfert, H. M. (2018). The first line of defence: insights into mechanisms and relevance of phagocytosis in epithelial cells. *Seminars in Immunopathology*, *40*(6), 555. https://doi.org/10.1007/S00281-018-0701-1

Hancock, R., & Fitz-James, P. C. (1964). SOME DIFFERENCES IN THE ACTION OF PENICILLIN, BACITRACIN, AND VANCOMYCIN ON BACILLUS MEGATERIUM. *Journal of Bacteriology*, *87*(5), 1044. https://doi.org/10.1128/JB.87.5.1044-1050.1964

Hunstad, D. A., & Justice, S. S. (2010). Intracellular lifestyles and immune evasion strategies of uropathogenic Escherichia coli. *Annual Review of Microbiology*, *64*, 203–221. https://doi.org/10.1146/ANNUREV.MICRO.112408.134258

JJ, M., MA, M., JD, S., JS, P., & SJ, H. (2000). Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. *The EMBO Journal*, *19*(12), 2803–2812. https://doi.org/10.1093/EMBOJ/19.12.2803

Kai-Larsen, Y., Lüthje, P., Chromek, M., Peters, V., Wang, X., Holm, Å., Kádas, L., Hedlund, K. O., Johansson, J., Chapman, M. R., Jacobson, S. H., Römling, U., Agerberth, B., & Brauner, A. (2010). Uropathogenic Escherichia coli modulates immune responses and its curli fimbriae interact with the antimicrobial peptide LL-37. *PLoS Pathogens*, *6*(7), 1–16. https://doi.org/10.1371/JOURNAL.PPAT.1001010

Kannan, K., & Mankin, A. S. (2011). Macrolide antibiotics in the ribosome exit tunnel: species-specific binding and action. *Annals of the New York Academy of Sciences*, *1241*(1), 33–47. https://doi.org/10.1111/J.1749-6632.2011.06315.X

Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of Anaesthesiology, Clinical Pharmacology*, *33*(3), 300. https://doi.org/10.4103/JOACP.JOACP_349_15

Kerkez, I., Tulkens, P. M., Tenson, T., van Bambeke, F., & Putrinš, M. (2021). Uropathogenic Escherichia coli Shows Antibiotic Tolerance and Growth Heterogeneity in an In Vitro Model of Intracellular Infection. *Antimicrobial Agents and Chemotherapy*, *65*(12). https://doi.org/10.1128/AAC.01468-21

Kim, W. J., Shea, A. E., Kim, J. H., & Daaka, Y. (2018). Uropathogenic Escherichia coli invades bladder epithelial cells by activating kinase networks in host cells. *The Journal of Biological Chemistry*, *293*(42), 16518. https://doi.org/10.1074/JBC.RA118.003499

Krause, K. M., Serio, A. W., Kane, T. R., & Connolly, L. E. (2016). Aminoglycosides: An Overview. *Cold Spring Harbor Perspectives in Medicine*, *6*(6). https://doi.org/10.1101/CSHPERSPECT.A027029

Lenz, K. D., Klosterman, K. E., Mukundan, H., & Kubicek-Sutherland, J. Z. (2021). Macrolides: From Toxins to Therapeutics. *Toxins*, *13*(5). https://doi.org/10.3390/TOXINS13050347

Levin-Reisman, I., Brauner, A., Ronin, I., & Balaban, N. Q. (2019). Epistasis between antibiotic tolerance, persistence, and resistance mutations. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(29), 14734–14739. https://doi.org/10.1073/PNAS.1906169116

Lewis, K. (2010). Persister cells. *Annual Review of Microbiology*, *64*, 357–372. https://doi.org/10.1146/ANNUREV.MICRO.112408.134306

Lüthje, P., & Brauner, A. (2014). Virulence factors of uropathogenic E. coli and their interaction with the host. *Advances in Microbial Physiology*, *65*, 337–372. https://doi.org/10.1016/BS.AMPBS.2014.08.006

Manfredi, R. (2006). Update on the appropriate use of linezolid in clinical practice. *Therapeutics and Clinical Risk Management*, *2*(4), 455–464. https://doi.org/10.2147/TCRM.2006.2.4.455

Mohanty, S., Kamolvit, W., Scheffschick, A., Björklund, A., Tovi, J., Espinosa, A., Brismar, K., Nyström, T., Schröder, J. M., Östenson, C. G., Aspenström, P., Brauner, H., & Brauner, A. (2022). Diabetes downregulates the antimicrobial peptide psoriasin and increases E. coli burden in the urinary bladder. *Nature Communications 2022 13:1*, *13*(1), 1–15. https://doi.org/10.1038/s41467-022-32636-y

Nelson, M. L., & Levy, S. B. (2011). The history of the tetracyclines. *Annals of the New York Academy of Sciences*, *1241*(1), 17–32. https://doi.org/10.1111/J.1749-6632.2011.06354.X

Nikaido, H. (2011). STRUCTURE AND MECHANISM OF RND-TYPE MULTIDRUG EFFLUX PUMPS. *Advances in Enzymology and Related Areas of Molecular Biology*, *77*, 1. https://doi.org/10.1002/9780470920541.CH1

Pahar, B., Madonna, S., Das, A., Albanesi, C., & Girolomoni, G. (2020). Immunomodulatory Role of the Antimicrobial LL-37 Peptide in Autoimmune Diseases and Viral Infections. *Vaccines*, *8*(3), 1–19. https://doi.org/10.3390/VACCINES8030517

Pakbin, B., Brück, W. M., & Rossen, J. W. A. (2021). Virulence Factors of Enteric Pathogenic Escherichia coli: A Review. *International Journal of Molecular Sciences*, *22*(18). https://doi.org/10.3390/IJMS22189922

Pandey, N., & Cascella, M. (2022). Beta Lactam Antibiotics. *Antibiotic Discovery and Development*, *9781461414001*, 79–117. https://doi.org/10.1007/978-1-4614-1400-1_3

PG, H., AC, F., & FJ, S. (2003). Fluoroquinolones: structure and target sites. *Current Drug Targets*, *4*(2), 181–190. https://doi.org/10.2174/1389450033346920

Poole, K. (2005). Efflux-mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy*, *56*(1), 20–51. https://doi.org/10.1093/JAC/DKI171

Prado-Silva, L., Cadavez, V., Gonzales-Barron, U., Rezende, A. C. B., & Sant'Ana, A. S. (2015). Meta-analysis of the effects of sanitizing treatments on Salmonella, Escherichia coli O157:H7, and Listeria monocytogenes inactivation in fresh produce. *Applied and Environmental Microbiology*, *81*(23), 8008–8021. https://doi.org/10.1128/AEM.02216-15/SUPPL_FILE/ZAM999116734SO1.PDF

R Snyderman, M. C. P. D. G. F. H. S. K. (1977). *Biologic and biochemical activities of continuous macrophage cell lines P388D1 and J774.1 - PubMed*. 119(6)-2060–2066. https://pubmed.ncbi.nlm.nih.gov/915291/

Ramirez, M. S., & Tolmasky, M. E. (2010). Aminoglycoside modifying enzymes. *Drug Resistance Updates*, *13*(6), 151–171. https://doi.org/10.1016/J.DRUP.2010.08.003

Rhee, K. Y., & Gardiner, D. F. (2004). Clinical relevance of bacteriostatic versus bactericidal activity in the treatment of gram-positive bacterial infections [2]. *Clinical Infectious Diseases*, *39*(5), 755–756. https://doi.org/10.1086/422881

Ronald, A. (2002). The etiology of urinary tract infection: Traditional and emerging pathogens. *American Journal of Medicine*, *113*(1 SUPPL. 1), 14–19. https://doi.org/10.1016/S0002-9343(02)01055-0

S Hayashi 1, S. O. Y. A. N. O. T. O. T. S. T. O. Y. N. (1987). *Human bladder carcinoma cell line HTB9, which secretes a factor to stimulate clonogenic leukemic blast growth, expresses the granulocyte-macrophage colony-stimulating factor gene - PubMed*. https://pubmed.ncbi.nlm.nih.gov/3121558/

Schmiemann, G., Kniehl, E., Gebhardt, K., Matejczyk, M. M., & Hummers-Pradier, E. (2010). The Diagnosis of Urinary Tract Infection: A Systematic Review. *Deutsches Ärzteblatt International*, *107*(21), 361. https://doi.org/10.3238/ARZTEBL.2010.0361

Shenouda, R., Abou El-Khier, N., El-Daker, M., Osman, Y., & Badr, R. (2020). Bacteriuria in Patients with Orthotopic Ileal Neobladder: The Role of Uropathogenic Escherichia coli. *Egyptian Journal of Medical Microbiology*, *29*(1), 87–93. https://doi.org/10.21608/EJMM.2020.249862

Sojo-Dorado, J., & Rodríguez-Baño, J. (2022). Gentamicin. *Kucers the Use of Antibiotics: A Clinical Review of Antibacterial, Antifungal, Antiparasitic, and Antiviral Drugs, Seventh Edition*, 964–991. https://doi.org/10.1201/9781315152110

Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. *American Journal of Infection Control*, *34*(5), S3–S10. https://doi.org/10.1016/J.AJIC.2006.05.219

Tenson, T., Lovmar, M., & Ehrenberg, M. (2003). The Mechanism of Action of Macrolides, Lincosamides and Streptogramin B Reveals the Nascent Peptide Exit Path in the Ribosome. *Journal of Molecular Biology*, *330*(5), 1005–1014. https://doi.org/10.1016/S0022-2836(03)00662-4

Wang, J., Shy, A., Wu, D., Cooper, D. L., Xu, J., He, H., Zhan, W., Sun, S., Lovett, S. T., & Xu, B. (2019). The Structure-Activity Relationship of Peptide Conjugated Chloramphenicol for Inhibiting E. coli. *Journal of Medicinal Chemistry*, *62*(22), 10245. https://doi.org/10.1021/ACS.JMEDCHEM.9B01210

Wei, X., Feng, Y., Chen, M., Zhang, S., Chen, M., Zhang, J., Zhang, Y., Ye, Q., Ding, Y., Xue, L., & Wu, Q. (2021). Recent Advances in Glycosidase Probes Used in Escherichia Coli Detection. *Current Medicinal Chemistry*, *28*(26), 5386–5410. https://doi.org/10.2174/0929867328999201224111615

Westblade, L. F., Errington, J., & Dörr, T. (2020). Antibiotic tolerance. *PLoS Pathogens*, *16*(10). https://doi.org/10.1371/JOURNAL.PPAT.1008892

Zalba, S., & ten Hagen, T. L. M. (2017). Cell membrane modulation as adjuvant in cancer therapy. *Cancer Treatment Reviews*, *52*, 48–57. https://doi.org/10.1016/J.CTRV.2016.10.008

NON-EXCLUSIVE LICENCE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC

I, **Turgay Hasanov,**

1. herewith grant the University of Tartu a free permit (non-exclusive licence) to

reproduce, for the purpose of preservation, including for adding to the DSpace digital archives until the expiry of the term of copyright,

Physiological heterogeneity of uropathogenic *Escherichia coli* in bladder epithelial cell infection model,

supervised by Ph.D. Marta Putrins, MSc Ivana Kerkez, and Prof. Dr.Tanel Tenson.

2. I grant the University of Tartu a permit to make the work specified in p. 1 available to the public via the web environment of the University of Tartu, including via the DSpace digital archives, under the Creative Commons licence CC BY NC ND 3.0, which allows, by giving appropriate credit to the author, to reproduce, distribute the work and communicate it to the public, and prohibits the creation of derivative works and any commercial use of the work until the expiry of the term of copyright.

3. I am aware of the fact that the author retains the rights specified in p. 1 and 2.

4. I certify that granting the non-exclusive licence does not infringe other persons' intellectual property rights or rights arising from the personal data protection legislation.

Turgay Hasanov

24/05/2023