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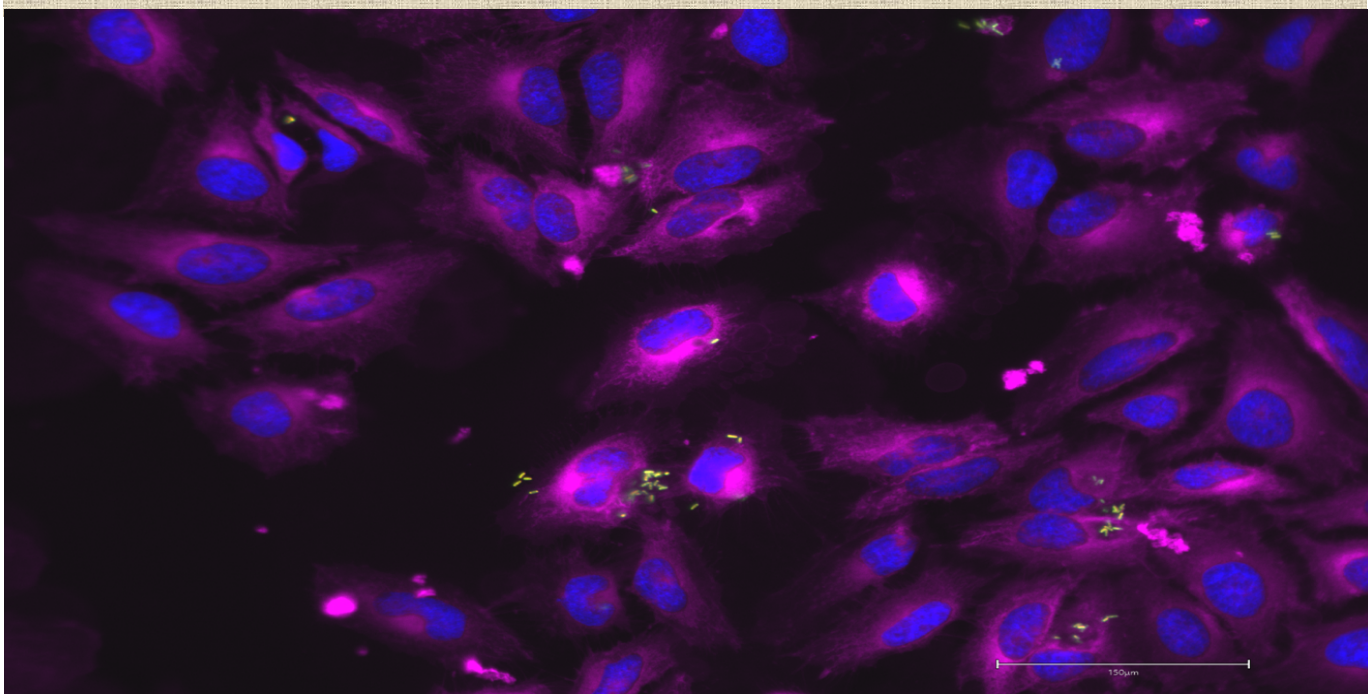
Núria Crua Asensio



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Department of Biochemistry, Molecular Biology and Biomedicine

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"So you can keep me inside the pocket of your ripped jeans
Holding me closer till our eyes meet
You won't ever be alone
Wait for me to come home"

"Luna piena, la tua buonanotte
Ma chi se ne frega di tutta sta gente
Che ci guarda come fossimo matti
Ma io vorrei fossimo sempre così"

"Per tu, pel padrí i per la iaia, ..."

Universitat Autònoma de Barcelona

DEPARTAMENT DE BIOQUÍMICA I BIOLOGIA MOLECULAR UNITAT DE BIOQUÍMICA DE BIOCIÈNCIES

Characterization of host-pathogen interactions during an infectious process

Thesis presented by **Núria Crua Asensio** to obtain the degree of Doctor in Biochemistry, Molecular Biology and Biomedicine under the supervision of Dr. Marc Torrent Burgas

Unitat de Biociències del Departament de Bioquímica i Biologia Molecular. Universitat Autònoma de Barcelona

Dr. **Marc Torrent Burgas** **Núria Crua Asensio**

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LIST OF PAPERS INCLUDED IN THE THESIS:

1. **Crua Asensio, N**., Muñoz Giner, E., de Groot, N. et al. Centrality in the host–pathogen interactome is associated with pathogen fitness during infection. Nat Commun 8, 14092 (2017). https://doi.org/10.1038/ncomms14092

2. **Crua Asensio, N.,** Macho Rendón, J., & Torrent Burgas, M. (2021). Time-Resolved Transcriptional Profiling of Epithelial Cells Infected by Intracellular Acinetobacter baumannii. Microorganisms, 9(2), 354.https://doi.org/10.3390/microorganisms9020354

Most of the experimental work of this thesis was performed at the System Biology of infection laboratory in the Universitat Autònoma de Barcelona (UAB). During the PhD a 3-month stay in 2020-2021, was spent at the Istituto Italiano di Tecnologia (Genova, Italy), under the supervision of the professor Gian Tartaglia and Elsa Zacco.

INDEX

SUMMARY

[CATALÀ]

Els continguts d'aquesta tesi estan enfocats en les interaccions entre un hoste-patogen durant un procés infectiu, per poder arribar a millorar el coneixement sobre les infeccions bacterianes i donar un punt de vista diferent per al disseny i síntesi d'antibiòtics.

Primerament, vam fer un estudi comparatiu de l'interactoma de *Yersinia pestis-Homo sapiens* i el *Yersinia pestis*, on vam poder relacionar que el número d'interaccions d'una proteïna en l'interactoma *Y.pestis-Homo sapiens* es pot relacionar amb la importància d'aquella proteïna, essent així més essencials les que tenen més interaccions. A més a més, aquest resultat es pot associar a la regla "*Centrality-lethality*", que diu que les proteïnes més connectades en una cèl·lula són les més importants per a la seva supervivència. Seguidament es van fer diferents estudis en els quals es suprimia un nombre determinat de nodes i s'observava els canvis en la xarxa d'interaccions hoste-patogen, que ens va permetre correlacionar que les proteïnes altament connectades també tenen un alt impacte amb la supervivència de l'organisme durant la infecció. Aquesta relació obre una nova visió sobre l'estudi de les interaccions proteïna-proteïna durant un procés infectiu, que a la llarga es pot fer servir per al desenvolupament de nous fàrmacs.

Per altra banda, s'ha estudiat les interaccions proteïna-proteïna durant un procés infectiu de cèl·lules epitelials humanes i el bacteri *Acinetobacter baumannii*, ja que els darrers anys aquest bacteri s'ha convertit en un bacteri amb una alta capacitat d'adquirir resistència als antibiòtics i causar moltes infeccions adquirides en els hospitals. Per això, es considerà molt important conèixer com aquest bacteri és capaç de sobreviure dintre d'una cèl·lula hoste.

En primer lloc, vam voler comprovar la capacitat del bacteri de sobreviure i replicar dins de la cèl·lula hoste, i a més a més, vam validar la seva capacitat de controlar-la. En comprovar que aquest bacteri és capaç d'infectar i penetrar la cèl·lula hoste ens fa veure clarament avantatges davant de la resistència als antibiòtics actuals. Seguidament, vam voler estudiar les interaccions proteïna-proteïna per saber quines són les proteïnes que més interaccionen d'*Acinetobacter baumannii* que en un futur es podrien utilitzar com a diana pel disseny de nous medicaments.

Com a conclusió general, aquesta tesi pretén donar noves perspectives sobre les malalties infeccioses. Estudiant les interaccions proteïna-proteïna durant la infecció, podem obrir nous camps de recerca i millorar el coneixement de les infeccions.

SUMMARY

[ENGLISH]

This thesis is focused on interactions between a host-pathogen during an infection process to improve the knowledge about bacterial infections and give a different perspective on the design and synthesis of new antibiotics.

First, we compare *Yersinia pestis-Homo sapiens* with *Yersinia pestis* interactome and we could relate the number of interactions of a protein with the percentage of essential protein. So, the proteins with more interacting nodes are the more essential proteins in the host-pathogen interactome. Moreover, this result can be associated with the "Centrality-lethality" rule, which states that the most connected proteins in a cell are the most important for their survival.

Finally, we attack a particular node of the host-pathogen interactome to see the effect in the host-pathogen network. From these calculations, we revealed that a centrality-based attack was the most effective in disrupting the global topological efficiency. This conclusion allowed us to correlate that highly connected proteins also have a high impact on the fitness of the organism during infection. This relationship opens up a new perspective on the study of protein-protein interactions during an infectious process, which can eventually be used for the development of new drugs.

On the other hand, we studied protein-protein interactions during an infectious process of human epithelial cells and Acinetobacter baumannii. Recently, this bacterium has emerged as one of the most important drug-resistance responsible for hospital-acquired infections. Therefore, there is an urgent need to study and understanding how this bacterium can survive inside a host cell. Initially, we proved the ability of the bacterium to survive and replicate inside the host cell. In addition, we validated its ability to control the host cell. *A.baumannii* has an intercellular lifestyle that gives clear advantages over antibiotic resistance. Later, we determined the protein-protein interactions network to identify hub proteins and in the future, can be used as a target for developing new antimicrobials.

As a general conclusion, this thesis aims to give new perspectives on infectious diseases. By studying protein-protein interactions during infection, opening up new fields of research, and understand infections at different levels of complexity.

INTRODUCTION

1. Introduction to bacterial infection

Bacteria are ubiquitous and play an important role in maintaining the environment in which we live. Although there are a great number of bacteria in and on our bodies, these bacteria normally do not cause disease. In fact, the bacteria in our digestive tract help us digest our food [1,2]. Sometimes, a small percentage of bacteria can cause infection and disease. An infection occurs when another organism enters the body and begins to multiply, causing a disease. The disease occurs when the host cells are damaged as a result of this infection, and different signs and symptoms of the illness appear [2-4]. As we know, these bacterial infections have a large impact on public health [5,6].

Adhesion of the pathogen to host cells is an essential preliminary step to start colonization and penetrate through tissues [7,8]. This step is also necessary to avoid innate physical host defense mechanisms, such as peristalsis in the gut and the flushing action of mucus, saliva, and urine. Successful adhesion and colonization also require that bacteria acquire essential nutrients, so the bacteria hijack host cells to their own benefit and start self-replication inside host cells [9,10]. In response to infection, the immune system springs into action, and white blood cells, antibodies, and other mechanisms start to activate the host body's defenses. Indeed, many of the symptoms that cause a person to suffer during infection, such as fever, headache, and rash, result from the immune system trying to eliminate the infection from the body [11].

Almost all infections are resolved by the action of the host's immune response, but sometimes bacteria multiply so rapidly that they crowd out host tissues and disrupt normal function. In this situation, the adaptive response is not enough to eradicate the infection, and the administration of antibiotics is required [12]. Bacterial infections are usually easy to treat with antibiotics. However, pathogens can adapt to drugs and develop new strategies to avoid the effects of antibiotics, thus becoming resistant bacteria. The resistance to antimicrobials is a rapidly growing problem with potentially devastating consequences [13,14].

1.1 How bacteria infect host cells

An infection occurs when a pathogen enters the body, increases in number, and causes an immune reaction. Accordingly, when a pathogen attaches to a host cell, it is considered the start of the infection process [15]. The majority of pathogenic bacteria are excluded from the host tissue by physical barriers that defend both the skin and mucous membranes. Skin is one of the body's first lines of defense against harmful bacteria. Specialized immune cells within the skin tissue and mucosal surfaces help fight invading organisms. The mucosal surfaces are protected by the active removal of bacteria, for example, by the acidic environment of the stomach, the ciliary movement in the upper respiratory tract, and the continuous flushing of urine in the lower urinary tract [9,15,16].

For that reason, motility and attachment factors found in pathogenic bacteria are essential for approaching cellular surfaces and adhering to host surfaces. It is important to recall that adhesion is considered a crucial step in host colonization [7,8,17]. One of the mechanisms of bacterial adherence is mediated by cell-surface structures called pili or fimbria. These structures are polymeric hair-like organelles protruding from the surface of bacteria that allow contact between the bacteria and the host cell. This interaction is normally mediated by a specific receptor architecture on host cell surfaces called adhesins. Adhesins are specialized proteins that mediate bacterial adhesion to host cells, recognize specific targets on the surface of host cells, and determine the tissue tropism of the pathogen [18-21].

Consequently, bacteria have developed a large arsenal of bacterial surface factors with adhesive properties, such as integrins, cadherins, and the immunoglobulin superfamily [22]. These host-pathogen interactions are specific; each bacterial species and tissue tropism have a different mechanism and involve the recognition of conserved microbial components, known as pathogen-associated molecular patterns (PAMPs), by the host pattern recognition receptors (PRRs) to activate the host cell immune response [23,24].

One of the most studied adhesins is the type 1 pilus adhesin *FimH*, which mediates attachment to α-D-mannosidases on the bladder surface and mediates colonization of the bladder in *Escherichia coli* urinary tract infections [25]. Moreover, Krebs, S. J. and Taylor, R. K. validated that the lack of expression of the toxin-coregulated pili in *Vibrio cholerae* significantly reduced the severity of bacteria-induced diarrhea in humans [26]. As an alternative, other mechanisms are being investigated, such as the secretion of bacterial toxins to facilitate colonization. *Bordetella pertussis*, the agent that causes whooping cough, paralyzes the ciliary clearance function of the respiratory tract via the release of cell wall constituents that induce nitric oxide-mediated ciliostasis [27].

Once the pathogen has adhered to the host cell, the next step is endocytosis, which consists of the internalization of the bacterium inside the host cell to evade the humoral immune response and proliferate in a well-protected environment [28]. Host-pathogen binding triggers bacterial internalization mediated by actin cytoskeleton rearrangements that trigger their own uptake by host cells [29]. As a result of the union, cell signals modulate gene expression to adapt to the cellular phenotype and activate phagocytosis. Phagocytosis is a fundamental cellular process through which eukaryotic cells can bind and engulf particles with their cell membrane. Particle engulfment involves cell-surface receptor recognition of specific particles, signaling, and remodeling of the actin cytoskeleton to guide the membrane around the particle.

Two main mechanisms of entry and membrane rearrangement are involved in this case: the zipper and trigger mechanisms *(Figure 1)*. Both activate signaling cascades, leading to the reorganization of the actin cytoskeleton at the level of the host plasma membrane [29,31].

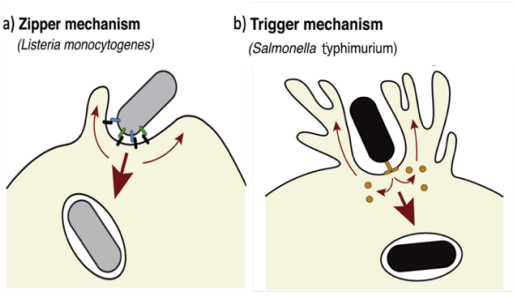

Figure 1. **Main mechanisms of bacteria entry to host cell. a**) Schematic representation of internalization of *Listeria monocytogenes* via the zipper mechanism, where engagement of bacterial surface proteins with host proteins induces cytoskeleton and membrane rearrangements, leading to the internalization of the bacterium. **b**) Representation of the trigger mechanism by the internalization of *Salmonella typhimurium* injection of effectors by the bacterium in the host cell cytoplasm triggers large-scale cytoskeletal rearrangements and ruffle formation, allowing the bacterium to be engulfed and internalized. Taken and modified from Cossart, P., & Helenius, A. (2014). Endocytosis of viruses and bacteria. Cold Spring Harbor perspectives in biology, 6(8), a016972.

The zipper mechanism is a type of entry with a specific interaction between host and pathogen. It activates host cell signaling pathways that result in moderate actin cytoskeleton rearrangements that wrap the bacterium [29,31]. For example, *Listeria monocytogenes* has evolved two main invasion proteins, called internalin and InlB. Internalin interacts with the cell adhesion molecule E-cadherin, and InlB mainly interacts with Met, the hepatocyte growth factor receptor undergoing successive post-translational modification (phosphorylation, ubiquitination), allowing the bacteria to invade the host cell [32].

On the other hand, in the trigger mechanism, the bacteria are injected directly into the host cell cytosol via a Type III secretion system (T3SS). At the same moment, many effectors are released, and different signaling cascades are activated to control host cytoskeleton dynamics and produce large membrane ruffles, which are responsible for the entry of the bacterium into the host cell [29,31]. *Salmonella* and *Shigella* are bacteria that use the trigger mechanism. Interestingly, bacterial effectors, such as *Salmonella SopB*/*SigD* or *Shigella IpgD*, are phospholipid-modifying enzymes that change membrane composition to achieve bacterial engulfment and *IpaA* induces actin de- polymerization at subsequent stages of the entry process [33,34].

Once the bacteria have completed the endocytosis and can be found inside the host cell, the intracellular bacteria have to start their own replication. Three main classes of compartments are known to be suitable for bacterial replication: lysosome-like vacuoles, intracellular non-acidic vacuoles, and directly on the cytosol (*Figure 2*) [29,7]. The first-class lysosome-like vacuoles have an acidic pH and contain hydrolytic enzymes. A well-known example of an intracellular bacterium is *Coxiella burnetti,* the causal agent of Q fever. Following internalization, the *Coxiella*-containing phagosome develops into a parasitophorous vacuole harboring lysosomal properties such as acidic pH, hydrolases, and cationic peptides. Despite difficult environmental conditions, *Coxiella* can replicate inside this compartment [35].

The second type, intracellular non-acidic vacuoles, do not fuse to lysosomes and are usually remodeled by the pathogen. In particular, pathogens are able to hijack the properties of these vacuoles by altering their protein and lipid compositions for their own benefit. *Salmonella*, for example, resides in vacuoles that undergo acidification but do not behave as lysosomes. Several effectors secreted by the T3SS of *Salmonella* locally remodel the actin cytoskeleton and regulate bacterial virulence [36]. Other effectors described can block the recruitment of nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase, which is responsible for the production of bactericidal compounds that normally kill intracellular bacteria [37].

Finally, the third class is the cytosol, in which some pathogens can reside after escaping from their internalization vacuole. *Listeria,* for example, can escape from the vacuole to arrive in the host cell cytosol using a pore-forming toxin secreted by the bacteria and two bacterial phospholipases, allowing the replication of the bacteria in the cytosol and movement into the cells by actin-based motility [38].

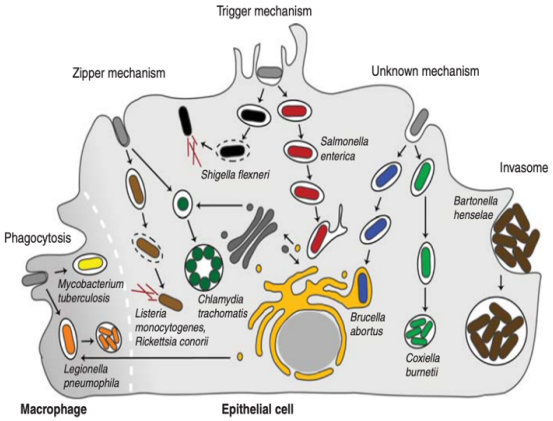

Figure 2. **Different representations of the mechanism of endocytic entry and replication by pathogenic bacteria.** Schematic representation of bacterial endocytosis mechanisms and intravacuolar and intracytosolic lifestyles. *Listeria monocytogenes* uses two different mechanisms to enter; then it resides temporally in a vacuole that later is lysed, allowing *L. monocytogenes* intracytosolic replication and actin-based motility. *Chamydia trachomartis* survives inside a vacuole that intercepts the shingomyelin transport from the Golgi apparatus. *Shigella flexneri* attaches to a filopodium found on the surface of epithelial cells. These filopodia are T3SS and are used to inject different effectors that promote the entry and formation of the vacuole. Later, *S. flexneri* is found in the intracytosolic area, where it replicates. Another example is *Salmonella enterica,* which uses T3SS-1 to control the entry and formation of vacuoles by effectors. Then *S. enterica* can replicate inside the vacuole, acquiring markers of endosomes and lysosomes. The *Brucella abortis* replicative vacuole is derived from the endocytic vacuole and matures in an endoplasmic reticulum (ER)-derived vacuole. Another mechanism is the ability to survive and replicate in a lysosome-derived vacuole, such as *Coxiella burnetii*. *Bartonella henselae* can lead to the formation of an invasome. Legionella and *Mycobacterium tuberculosis* reside in vacuoles formed by macrophages. The *Legionella pneumophila* vacuole acquires markers of the ER. On the other hand, *M. tuberculosis* blocks the maturation of the internalization vacuole. Taken and modified from Cossart, P., & Helenius, A. (2014). Endocytosis of viruses and bacteria. Cold Spring Harbor perspectives in biology, 6(8), a016972.

Among the different advantages conferred by each type of intracellular lifestyle are the internalization and replication of pathogens that facilitate the dissemination of bacteria in their host. Dissemination is how a pathogen is able to reach other sites or another host to start replicating again. These interactions and dissemination define the severity of the disease and the outcome of infection. Thus, determining how hosts and pathogens interact during an infection is key to understanding the disease and designing new strategies to control it [7,8].

1.2 Bacteria biofilms

Biofilms are populations of microorganisms that coexist to constitute a protected mode of growth thanks to an extracellular polymeric matrix (EPM). This EPM matrix surrounds the bacteria, creating a protective environment in which bacteria can survive and become more resistant to host defenses and antibiotic treatments [39,40].

The most frequent microorganisms associated with the biofilm strategy are gram-positive bacteria, *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Enterococcus faecalis,* and gram-negative bacteria, including *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Proteus mirabilis,* and *Pseudomonas aeruginosa* [41].

In general, biofilm formation is a four-stage process that begins with the adherence of the bacteria to a substrate surface, continues with the proliferation of the attached bacteria and the formation of the EPM, progresses with the maturation of the attached cells, and is followed by a detachment of the biofilm. From a molecular biology point of view, these stages are mainly controlled by surface adhesins and cell-to-cell communication signaling pathways. Therefore, the adhesion of bacteria to host surfaces is a key element in the formation of biofilms [39,40].

As a general idea of the composition of biofilms, the extracellular matrix is composed of polysaccharides, proteins, extracellular DNA, and lipids, which are separated by water channels, allowing the flow of nutrients, oxygen, and microorganisms into the microbial community [39-41]. There is intercellular signaling between bacteria that belong to the community, which is referred to as quorum sensing (QS). This communication allows for synchronizing and alteration in the genetic expression of the whole bacterial population, thus coordinating activities such as biofilm formation and the production of virulence factors [42].

Although several QS systems are known, perhaps the two most thoroughly described systems are acyl-homoserine lactone (acyl-HSL) and peptide-based signaling. Gram-negative bacteria use the production of N-acylhomoserine lactones (AHL) as QS molecules. Once a certain concentration is reached, AHL binds to a cytoplasmic transcription factor that binds directly to bacterial DNA and regulates genetic transcription [43,44].

As a result, this EPM creates an environment that helps bacteria survive, and the antibiotic loses its effects because it must cross the extracellular biofilm matrix to reach the embedded bacteria. In the majority of cases, antibiotics are inefficient on biofilms [45,46]. Consequently, bacterial biofilms have several health impacts [47]. Biofilms may be present anywhere, but the most commonly described ones develop chronic bacterial infections in kidney stones, dental plaques, and chronic inflammatory infections. The infection of implantable medical devices can have catastrophic complications, and the consequences of implant removal are very large in terms of morbidity, mortality, and financial burden [48]. One of the most well-known examples is chronic *Pseudomonas aeruginosa* lung infections in cystic fibrosis (CF) patients caused by biofilm-growing mucoid strains [49].

1.3 Host response to an infection

At the same moment that the pathogen is attached, the host response is activated. The first challenge encountered by bacteria is the innate immunity defense, which includes the barrier function of the skin and mucous membranes [50]. The skin is formed by an outer layer of cells that produce keratin and fatty acids and prevent bacterial adhesion. Mucous is found on many body surfaces because the sticky property of mucus provides a physical trap for pathogens, preventing their movement deeper into the body [51]. The stomach has the ability to create a highly acidic environment by controlling pH, which kills many pathogens entering the digestive system. Also, ears and noses are protected by hairs that catch pathogens [52].

Another interesting host defense strategy is the production of antimicrobial peptides (AMPs). During the co-evolution of host-pathogen interaction, AMPs are used as a natural component of system defense. They are known to boost immunity by working as immunomodulators. So, AMPs can protect the host against many types of pathogens [53].

Once the bacteria are detected by the host cell, many types of epithelial cells are activated and have the intrinsic ability to recognize and identify the pathogen-host interactors to respond specifically through the activation of innate immunity. This activation is mediated by the detection of bacterial PAMPs and the specific host cell receptor [54]. These well-conserved bacterial structures are recognized by different types of innate immune defense cells, such as blood monocytes, tissue macrophages, dendritic cells (DCs), and natural killer cells. Many types of bacterial PAMPs have been characterized, such as the bacterial cell envelope, lipopolysaccharide (LPS), peptidoglycan, toxins, and bacterial DNA. These PAMPs are detected normally for toll-like receptors and nucleotide-binding and oligomerization domain-like receptors, which are known for their role in cellular signaling and the initiation of the adaptive immune response [55].

As a result of the interaction between PRRs and PAMPs, a cascade of cytokines and chemokines is released to trigger innate immunity. The innate immunity represents the first line of host defense, and it is a non-specific defense. The stimulation is mediated by the cytokines of the interleukin family (IL-1, IL-6), the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), and the mitogen-activated protein kinase, which upgrade the innate immune system to an inflammatory response [50, 56].

The inflammatory response is responsible for the recruitment of various cell lineages, which play a crucial role in early pathogen elimination and also represent an important link from the adaptive immune response to a major signal transduction pathway. These required cells detect potential harmful pathogens and inform the adaptive immune response. The antigen-presenting cell (APC) phagocytoses the pathogen and digests it to obtain many fragments of the pathogen and produce antigens. These antigens are transported to the APC surface, where they serve as an indicator to other immune cells to activate the adaptive immune response [57].

When APC cells present bacterial antigens, the adaptative immune response is activated and replayed, activating an antigen-specific reaction through T lymphocytes and B lymphocytes. Whereas the innate response is rapid but not specific, the adaptive response is precise and takes several days or weeks to develop. The most relevant part of adaptive immunity is the capacity to obtain memory, which enables the host to mount a more rapid and efficient immune response upon subsequent exposure to the antigen [57,58].

There are different pathways controlled by the adaptive system: the cell-mediated immune response, which is carried out by T cells, and the humoral immune response, which is controlled by activated B cells and antibodies. Three types of T cells—cytotoxic, helper, and suppressor T cells—are key components in the cell-mediated response and neutralize cells that have been infected with bacteria. Cytotoxic T cells destroy bacteria-infected cells; helper T cells play a part in activating both the antibody and the cell-mediated immune responses; and suppressor T cells deactivate T cells and B cells when needed, thus preventing the immune response from becoming too intense. Memory cells persist after primary exposure to a pathogen. If re-exposure occurs, memory cells differentiate into effector cells without input from the innate immune system and eradicate the pathogen much faster than they do in an initial exposure [57-59].

Currently, bacteria are evolving new strategies to prevent and avoid the immune system response, and each day, the administration of antibiotics is increasingly required to eradicate these infections. As we know, each time the administration of antibiotics is required, bacteria adapt to the antibiotic and learn how to avoid its effects to continue growing and spreading [60]. Therefore, a better understanding of the mechanisms of infection and system immunity will lead to new avenues for the eradication of pathogens. Is important to highlight that AMPs are gaining strength as potential therapeutic agents; some studies verify that AMPs can combat antibiotic-resistant bacteria, including biofilms [61].

1.4 Intracellular survival and evasion of the immune system

Bacteria have numerous sophisticated mechanisms to counteract the immune system. Each time bacteria develop highly effective mechanisms and strategies for avoiding or inactivating the human immune system defenses and ensuring their survival inside a host. Pathogens employ tactics such as modulating their own cell surfaces, releasing proteins to inhibit or degrade host immune factors, or even simulating and mimicking host molecules. All these strategies and techniques by bacterial pathogens complicate efforts to develop new vaccines and innovative treatments [62,63].

The most effective strategy for avoiding innate recognition could be the modification of exposed PAMPs. Studies suggest that some gram-negative bacteria, such as *E. coli* and *S. enterica,* acylate the lipid A component of the lipopolysaccharide in their cell walls, thereby changing their negatively charged surface to a positive charge and allowing the bacterial cells to repel positively charged AMPs produced by host immune cells [64]. Some bacteria have developed an efficient way to control intracellular phagocytic cells for their own benefit. As a result, the pathogen uses

a variety of strategies to avoid engulfment and degradation by phagocytes and to facilitate proliferation and spread among host tissues. For example, *Yersinia* species use their T3SS to inject several T3SS effectors that effectively neutralize phagocytic activity [65,66].

As mentioned in Section 1.1, bacteria can enter the host cell by different mechanisms, and each type of bacteria acts differently. Bacteria search for intracellular compartments in which to grow and replicate under optimal conditions. The majority of intracellular bacteria remain in the lumen of endocytic vacuoles, which they modify to provide maximal protection and support [67] but the properties of bacteria-containing vacuoles differ greatly between bacteria. Each bacterial species has developed sophisticated mechanisms to ensure the remodeling of the vacuolar environment, allowing it to survive and replicate. Also, some bacteria devise strategies to ensure that the vacuoles do not mature to a point at which they fuse with lysosomes. Yet, inside cells, pathogens have to confront a variety of host cellular defense mechanisms.

2. Antibiotic resistance

Unfortunately, sometimes immune system defense is not enough to stop infections caused by bacteria, and the administration of antibiotics is required to help treat bacterial overgrowth in host cells [13]. Antibiotics are drugs used to treat infectious diseases that have the capacity to inhibit bacterial growth. Antibiotics are specific, and each type of bacteria requires a specific type of antibiotic. The administration of antibiotics usually does not have many side effects, but in some cases, such as infants or patients with kidney or liver disease, the administration needs to be controlled and specify the type and dose required [68,69].

How antibiotics act against pathogens can be explained using the mechanism of action of each type of antibiotic, that is—from a biochemical view—how a drug is pharmacologically effective. There are many types of antibiotics, but antibacterial action generally falls within one of five mechanisms. Three involve the inhibition or regulation of cell wall biosynthesis, protein synthesis, or nucleic acid synthesis. The fourth mechanism involves the disruption of the membrane structure, and the last one alters antimetabolite activity [70,71].

In 1928, penicillin was the first commercialized antibiotic discovered by Fleming. Since then, the discovery of new antibiotics has decreased each year and has been marred by the emergence of hard-to-treat multiple antibiotic-resistant infections [13]. Even before penicillin was introduced, resistant strains of bacteria were detected. The low rate of antibiotic discovery, coupled with the rapid spread of drug-resistant bacterial pathogens, is causing a global health crisis. Antibiotic resistance occurs when a drug loses its ability to inhibit bacterial growth effectively. Bacteria become *resistant* and can replicate in the presence of antibiotics [68].

A significant overuse of antibiotics in recent years is the reason that these drugs are becoming less effective and has led to the emergence of superbugs. The world urgently needs to change the way it prescribes and uses antibiotics and reduce the number of antibiotics used in hospitals, the community, and agriculture. All of these points have contributed to the selection pressure of

bacteria-resistant strains, forcing a shift toward a more expensive and broader-spectrum antibiotic [72].

Other causes of antibiotic inefficiency are the inappropriate prescription of antibiotics and the administration to farm animals as growth supplements. Recent studies have shown that treatment indication, choice of agent, or duration of antibiotic therapy is incorrect in 30–50% of cases in the U.S. An estimated 80% of antibiotics sold in the U.S. are used in animals to prevent infections. As a consequence, antibiotics used in livestock are ingested by humans when they consume food, and the transfer of resistant bacteria to humans by farm animals occurs [69,73].

Resistance occurs naturally, but there is clear exploitation of antibiotics by humans, provoking acceleration of the process. Antibiotic resistance generates longer hospital stays, higher medical costs, and increased mortality. Each year in the U.S., at least 2.8 million people are infected with antibiotic-resistant bacteria or fungi, and more than 35,000 people die as a result. In Europe, over 33,000 people die as a consequence of bacterial infections [69,73]. The U.S. Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) categorize antimicrobial-resistant (AMR) pathogens as imminent threats to human health. In addition, the development of new antibiotics by the pharmaceutical industry has decreased due to economic and regulatory obstacles. Nowadays, the number of active programs to develop and test new antibiotics is scarce. One of the main reasons why pharmaceutical companies lose their interest in producing new antibiotics is that antibiotics are used for relatively short periods, are often curative, and are relatively inexpensive, so they are not as profitable as drugs that treat other chronic illnesses [69,73].

The distribution of resistance genes, such as *Enterobacteriaceae* producing extended-spectrum β-lactamase, New Delhi metallo-β-lactamase-1 and *Klebsiella pneumoniae* carbapenemase, indicates how resistance can spread [74,75]. Carbapenem resistance among *Enterobacteriaceae* has increased significantly over the past decade. Is evident that there is a huge decreasing effect of antibiotics in treating resistant infections [76,77].

The most commonly reported resistant bacterial species are *E. coli* (44.2%), followed by *S. aureus* (20.6%), *K. pneumoniae* (11.3%), *Acinetobacter* species (1.7%), *P. aeruginosa* (5.6%), *E. faecalis* (6.8%) and *E. faecium* (4.5%). These bacteria coincide with the pathogens in the acronym ESKAPE, which refers to their ability to evade the effects of commonly used antibiotics [78,79]. These pathogens have developed resistance mechanisms against a large list of antibiotics, such as oxazolidinones and clinically unfavorable polymyxins [80].

In addition, a Priority Pathogens List was published by the WHO (2017) to promote the research and development of new antibiotics for these pathogens. *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacteriaceae* are on a critical bacterium list. The biggest problem is that new strains of emerging multidrug-resistant bacteria cannot be treated with any existing antibiotics. In this context, understanding how pathogen bacteria can penetrate and survive inside a host is therefore crucial to avoiding the development of antibiotic resistance [78-80].

2.1 Mechanisms of drug resistance

The main mechanisms of resistance are limiting uptake of a drug, modification of the target, inactivation of the antibiotic, active efflux of a drug, and biofilm formation. These mechanisms can be native to microorganisms or acquired from other microorganisms. Understanding more about these mechanisms will hopefully lead to better treatment options for infective diseases [81-83].

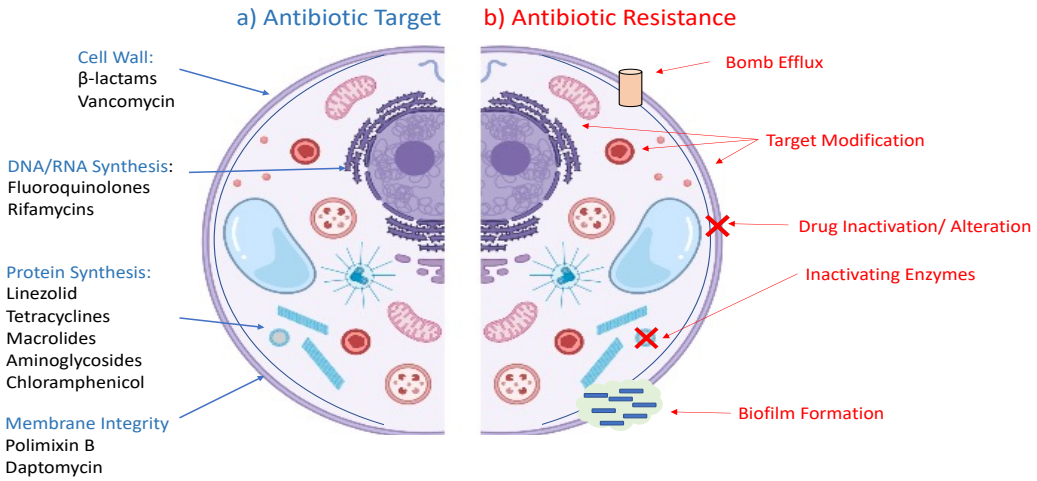

Figure 3. ***Antibiotic targets and mechanisms of resistance.*** **a**) A schematic representation of antibiotic targets can be divided into different groups depending on the mechanism of action, such as Cell Wall. Several classes of antibacterial block steps in the biosynthesis of peptidoglycan, making cells more susceptible to osmotic lysis. DNA/RNA Synthesis targets are a group of antibiotics that interfere with DNA synthesis by inhibiting topoisomerase and RNA synthesis by specifically inhibiting bacterial RNA polymerases. Protein Synthesis targets are a variety of broad-spectrum, bacterial protein synthesis inhibitors that selectively target the prokaryotic 70S ribosome, including those that bind to the 30S subunit (aminoglycosides and tetracyclines) and others that bind to the 50S subunit (macrolides, lincosamides, chloramphenicol, and oxazolidinones), and Membrane Integrity agents that target phospho-lipids and can alter membrane properties. One such way is to alter the bulk physical properties of the membrane. **b**) Resistance to antibiotics can be mediated by a range of mechanisms: Bomb Efflux activation to eliminate the drug, Target Modification when bacteria mutate the affinity of the drug for the target., Drug Inactivation / Alteration or Inactivating Enzymes, when drug loss the efficiency, Biofilm formation, when the bacteria grow as a community and synthetize a matrix that provide an antibiotic resistance.

a.) Drug inactivation/alteration

There are many ways to inactivate a drug. One example is the transfer of a chemical group to the drug, such as acetyl, phosphoryl, or an adenyl group. The most frequently used is acetylation, which is known to be used against aminoglycosides, chloramphenicol, streptogramins, and fluoroquinolones. Enzymes that irreversibly modify and inactivate antibiotics are also a mechanism to alter the antibiotics, such as β-lactamases, aminoglycoside-modifying enzymes, or chloramphenicol acetyltransferases. A clear example is β-lactamases, a group of enzymes that deactivate penicillin [81-84].

b.) Changes in cell permeability and active drug efflux result in reduced intracellular drug accumulation

Bacteria must balance the uptake and ability of the bacteria to get rid of the antibiotic and determine the susceptibility of the bacteria to a particular antimicrobial. The antibiotic passes through the bacterial cell membrane using porins and efflux pumps. These pumps remove the drug from the cell at a high rate, with the objective that the drug concentrations are never high enough to evoke an antibacterial effect.

The outer membrane proteins (OMPs) of gram-negative bacteria contain porin proteins that form channels, allowing the passage of many hydrophilic substances, including antibiotics. For example, multiple-drug-resistant *K. pneumoniae* exhibits susceptibility to β-lactams (such as cephalosporins and carbapenems) due to the loss of OMPs known as OmpK35 and OmpK36 [85].

c.) Modification of drug binding sites/targets

Another approach used by resistant bacteria is the modification of the target site of the drug. One known example is the mutation in the gene of penicillin-binding proteins (PBPs). *Staphylococcus aureus* expresses a unique PBP2a, which has a low affinity for all β-lactam antibiotics and acts as a substitute for the other PBPs, thus enabling the survival of *S. aureus* in the presence of high concentrations of β-lactam drugs, including methicillin, acting on cell wall biosynthesis [86].

d.) Biofilm formation

As mentioned previously, biofilms are complex microbial communities protected with an EPM, which generates a protective environment against different kinds of antibiotics. Biofilms are difficult to treat and normally require long-term therapy. Biofilm formation also enables bacteria to survive long exposures to antibiotics, and there is a high rate of horizontal gene transfer [43,86].

Bacterial biofilms have the ability to adapt to stressful conditions. For instance, organisms present in a biofilm increase their capacity to neutralize monochloramine, control the expression of chromosomal β-lactamases, or stimulate catalase production when they are exposed to prolonged treatment with a certain antibiotic. In addition, these communities can switch themselves to be more or less tolerant depending on environmental conditions, such as alterations in temperature, osmolarity, pH, cell density, and nutritional quality, by turning on stress-response genes. These stress-response genes can antagonize antibiotic effects and environmental toxins and can avoid the immune system. These genes are regulated by the interacting signals of QS [88,89].

2.2 New approaches to tackling the problem

One approach to tackling the problem of antibiotic resistance is the discovery of new molecules using bioinformatics design. Thanks to bioinformatics and *omics*, we can study libraries of molecules and see how a molecule's structure will affect its behavior to understand the structure-activity relationship. The data can help create virtual models to satisfy given functionalities (molecule size, overall charge and hydrophobicity, charge and binding to a specific target, etc.) and combine them to design a molecule [90,91].

A perfect example is linezolid, a synthetic antibiotic associated with a new class of antimicrobials called oxazolidinones. A synthetic antibiotic means that linezolid is synthesized de novo and does not occur in nature, so it was designed bioinformatically [92]. Linezolid is a protein synthesis inhibitor that affects the initiation phase of bacterial protein synthesis, providing a new way to overcome resistant bacteria [93]. This drug disrupts the initiation of protein synthesis and forms a new group of antibiotics because of its mechanism of action. It has been approved for certain gram-positive infections, including certain drug-resistant enterococcus, staphylococcus, and pneumococcus strains. It can be used as an oral therapy because it is well absorbed, in general, is well-tolerated, and the most serious adverse effect is related to myelosuppression. Today, this antibiotic can treat multidrug-resistant infections [94].

Every day, there are new approaches to fight against resistant bacteria; for example, nanoparticles/nanomaterials have emerged as promising alternatives with potential leads possessing enhanced antimicrobial activities or the use of AMPs. One other new idea to tackle the problem of biofilm formation is using QS inhibitors. It is well known that bacteria can survive for prolonged periods due to their communication. So, QS inhibitors work synergistically with antibiotics to eliminate biofilms [95,96].

In conclusion, host-pathogen interactions, cell attachment, or immunosuppression open new alternatives to produce drugs. All this information can provide a new generation of drugs with a long-lasting life that will hopefully overcome the current antibiotic crisis.

3. Overview of the principal bacteria examined

Despite new approaches to fighting resistant bacteria, we still need to obtain further information about bacterial infection. As mentioned in Section 2, the problem of nosocomial antimicrobial resistance has been mainly associated with a particular group of microorganisms—the ESKAPE bugs [79-80].

Many bacteria are susceptible to antibiotics, but the ESKAPE bug group, composed of *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species, has the ability to evade the effects of antimicrobial agents. The high prescription and inappropriate use of antibiotics promote the acquisition of resistance mechanisms by the bacteria, either by mutation events or horizontal gene transfer [78-80].

Each day, the ESKAPE pathogens increase in frequency and cause significant morbidity and mortality [80]. New antimicrobial agents are needed to treat these infections. We also wanted to add *Yersinia* species as the causes of different important diseases. *Yersinia pestis* is the cause of plague disease, a human disease that was very prevalent in Europe during the Middle Ages [97,98]. The transmission of the disease was usually by a rodent flea bite or by handling an animal infected with the plague. While modern antibiotics are effective against the plague, the disease has not yet been fully eradicated, and some infections still occur in the western United States, Africa, and Asia. The WHO has reported that yersiniosis is an important disease that is usually caused by eating raw or undercooked pork contaminated by an overgrowth of *Yersinia enterocolitica* bacteria. The CDC estimates that *Y. enterocolitica* causes 117,000 illnesses, 640 hospitalizations, and 35 deaths in the United States every year. Moreover, children are infected more often than adults, and infection is more common in winter.

3.1 Acinetobacter baumannii

Acinetobacter consists of short, gram-negative, aerobic, catalase-positive, oxidase-negative, nonfermenting, and nonmotile coccobacillus. Over the last few decades, *Acinetobacter baumannii* has emerged as one of the main pathogens responsible for hospital and community-acquired infections [99,100]. Its clinical significance has been driven by its remarkable ability to acquire resistance, making it one of the most successful multidrug-resistant (MDR) organisms in overcoming current antibiotic therapy [101-103].

A. baumannii can cause pneumonia, septicemia, meningitis, urinary tract and wound infections, and is associated with producing high mortality [104]. The WHO has assigned *A. baumannii* as a critical priority pathogen, as it is a great threat to human health and new antibiotics are urgently needed. In particular, *A. baumannii* has a small genomic region of 86 kilobases containing 45 resistance genes [99-101]. Thanks to the resistance genes, *A. baumannii* has become extremely resistant to several antimicrobial molecules, and there are only a few antibiotics that can eradicate

the infections it causes. Some strains are resistant to carbapenems and polymyxins, meaning that combination therapy is the final treatment option available [102-105].

a.) Adhesion and intracellular survival of *Acinetobacter baumannii* in host cells

Many studies have focused on the adherence of this pathogen to host cells to understand how several proteins are involved in this process. In this case, *A. baumanni* uses a zipper-like mechanism that requires a receptor-mediated mechanism with a direct interaction of the bacterial ligand, the host's cell-surface receptors, and local cytoskeletal rearrangement at the invasion site [106].

OmpA is a β-barrel porin highly conserved among bacterial species and in the case of *A.baumannii* has been associated with modulation of cellular permeability, bind host epithelia, target mitochondria, translocate to the nucleus, and induce cell death. For that reason, is considered a virulence factor of this bacteria [107]. Moreover, *A. baumannii* mutants that are defective in OmpA are unable to infect the host cells [108]. Other proteins, such as Phospholipase D (PLD) and Phospholipase C (PLC), have been described as virulence factors that are important for resistance to human serum, epithelial cell evasion, and toxicity [109]. Also, the trimeric autotransporter adhesin *Ata* has been described as a key effector to invade the eukaryotic host cells by *A. baumannii* [110].

How *A. baumannii* survives inside host cells is not clear. Choi et al. have suggested that these bacteria live within membrane-bound vacuoles in the cytoplasm, similar to other intracellular pathogens (e.g., *Neisseria*, *Listeria*, *Salmonella,* and *Yersinia*). However, studies of the intracellular lifestyle of *A. baumannii* are limited in number; for that reason, more research is required to understand the pathogenicity of this microbe.

The high ability of *A. baumannii* to form biofilms on surfaces can include glass and equipment used in intensive care units or epithelial cells. The most common factors that control biofilm formation are nutrient availability, the presence of pili and OmpA, and macromolecular secretions. Pili assembly and the production of biofilm-associated proteins contribute to the initiation of biofilm formation [111].

b.) Mechanisms of antibiotic resistance developed by *Acinetobacter baumannii*

A. baumannii has a native ability to adapt quickly to changes in environmental pressure. It is also important to highlight that an organism's regulation to generate new resistance mechanisms coupled with the acquisition of foreign resistance genes are crucial to becoming an MDR pathogen. As we mentioned before, this pathogen has a resistance *island* that contains a cluster of 45 resistance genes, which gives a high rate of versatility [99,102].

Current studies on the virulence factors of *A. baumannii* include porins, capsular polysaccharides, lipopolysaccharides, phospholipases, outer membrane vesicles, metal acquisition systems, and

protein secretion systems [100]. Mechanisms of antibiotic resistance of this organism include the acquisition of β-lactamases, up-regulation of multidrug efflux pumps, modification of aminoglycosides, permeability defects, and alteration of target sites [100,112].

On one hand, *Acinetobacter baumannii* intrinsically possesses a reduced susceptibility to antibiotics, which is related to the intrinsic two β-lactamase genes (one for AmpC and another for class D) whose expression can be modulated, together with efflux systems and permeability defects. On the other hand, aminoglycosides belong to antibiotic families that target bacterial translation, and multiple aminoglycoside-modifying enzymes (AMEs), including phosphotransferases, acetyltransferases, and adenyltransferases, have been reported in *A. baumannii* [100,112].

Quinolones and fluoroquinolones inhibit bacterial DNA replication by targeting DNA gyrase and DNA topoisomerase IV enzymes. The *ser-86-Leu* substitution in *gyrA*, together with the *Ser-80-Leu* substitution in *parC*, are commonly identified, significantly increasing the minimum inhibitory concentrations of ciprofloxacin [113]. Efflux-mediated resistance to quinolones has also been described as involving efflux pumps that are intrinsic in *A. baumannii*. These systems are able to pump out quinolones and therefore contribute to high-level resistance to these compounds. The main mechanisms of carbapenem resistance in *A. baumannii* correspond to the Ambler class D-carbapenem-hydrolyzing β-lactamases *OXA-23*, *OXA-40*, and *OXA-58*. However, the antibiotic resistance mechanisms remain very challenging, and colistin, tigecycline, and rifampicin often remain active against MDR *A. baumannii* [100,112].

3.2 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa (*family *Pseudomonadaceae)* is an aerobic, motile, gram-negative bacteria that can grow and survive in almost any environment. *P. aeruginosa* is considered an important cause of infections in hospitalized patients, immunocompromised hosts (e.g., HIV, cancer), and patients with CF [115]. The genome of *P. aeruginosa* (5.5–7 Mbp) is relatively large compared with other bacteria, such as *Bacillus subtilis* (4.2 Mbp), *Escherichia coli* (4.6 Mbp), and *Mycobacterium tuberculosis* (4.4 Mbp), and encodes many enzymes important for metabolism, transportation, and efflux of organic compounds [116].

This bacterium plays a prominent role in hospital infections and has revealed trends of increasing antimicrobial resistance, including carbapenem resistance and multidrug resistance. In Europe, a 2016 report by the CDC showed that 33.9% of *P. aeruginosa* were resistant to at least one of the antimicrobial groups. Interestingly, *P. aeruginosa* planktonic cells can be efficiently eliminated by different agents, such as carbapenems, fluoroquinolones, and aminoglycosides; however, these agents are not severe enough when *P. aeruginosa* is forming biofilms [117,118].

Pseudomonas aeruginosa has been recognized as a biofilm-forming pathogen that is commonly associated with nosocomial infections and ventilator-associated pneumonia. It rarely affects healthy individuals, but causes high morbidity and mortality in CF patients and immunocompromised individuals [119]. CF is a genetic disorder caused by mutations in the gene

encoding the cystic fibrosis transmembrane conductance regulator (CFTR) found on chromosomes. This CFTR mutation impairs cyclic AMP-dependent chloride ion transport across epithelial cell membranes. This reduction in chloride ion transport is associated with sodium and water hyperabsorption at epithelial surfaces, resulting in dehydrated mucus in the lung of a CF patient. This mutation creates the perfect environment for *P. aeruginosa* lung colonization [119-121].

This lung biofilm formation causes a chronic inflammatory state, and inflammation leads to airway damage manifesting as bronchiectasis, ultimately resulting in end-stage lung disease. CF patients suffer from persistent and recurrent lung infections caused by *Pseudomonas aeruginosa.* In addition, it is important to highlight the lack of treatment efficacy in antibiotic-resistant biofilms forming in the lungs of CF patients [119-121].

a.) Adhesion and intracellular survival of *Pseudomonas aeruginosa* in host cells

The molecular interactions of *P. aeruginosa* with host cells are complex and likely involve multiple types of ligand-receptor contacts that are poorly known. The attachment of *P. aeruginosa* to human respiratory mucus represents an important step in the development of lung infection, especially in CF [122]. Respiratory tract factors, such as osmolarity or iron concentration, might influence this colonization. *P.aeruginosa* encodes multiple adhesins that bind to different host cell types. The most important adhesins are Type IV pili, polar fimbriae that undergo extension and retraction, and flagella, which are polar organelles that also mediate swimming motility [123].

P. aeruginosa interacts with various host cell receptors, including $\alpha 5\beta 1$ integrin, CFTR, and the glycosphingolipids (GSLs): GM1 and GM2, to ensure efficient adhesion and cell entry. Although GM1 and GM2 are not directly involved in initial Type IV pili-mediated adhesion, it is widely accepted that host cell GSLs can be hijacked by microbial factors, leading to signaling and internalization events [124]. Different approaches have revealed that *P. aeruginosa* internalization requires rearrangement of the actin cytoskeleton through pathways involving Abl kinase, the adaptor protein Crk, the small GTPases Rac1 and Cdc42, and p21 kinase [125,126].

b.) Mechanisms of antibiotic resistance developed by *Pseudomonas aeruginosa*

P. aeruginosa is characterized by its notable intrinsic resistance to antibiotics. Treatment has become a great challenge due to the resistance of this bacterium to many of the currently available antibiotics [127]. Moreover, *P. aeruginosa* biofilm formation capacity is responsible for prolonged and recurrent infections in CF patients [128].

Eight different categories of antibiotics are mainly used to treat *P. aeruginosa* infections, including aminoglycosides, carbapenems, and polymyxins (colistin, polymyxin B). Generally, the major mechanisms of resistance of *P. aeruginosa* include the production of antibiotic-inactivating enzymes, the expression of efflux pumps that expel antibiotics out of the cell, target modification, and biofilm formation. It is also important to highlight the natural occurrence of mutations in the genome of bacteria, which perhaps in some cases can increase resistance, normally these

mutations do not lead to clinically problems although there is an accumulation of several mutations. In addition, some strains can acquire resistance via the horizontal transfer of genes, also known as lateral gene transfer is the process by which an organism incorporates genetic material from another organism [115,116,117,129,130].

Aminoglycoside-modifying enzymes inactivate aminoglycosides by the attachment of acetyl, phosphate, or adenyl groups to amino and hydroxyl substituents on the antibiotic molecule. These modifications reduce the affinity of aminoglycosides to the 30S ribosomal subunit target and block their activity [131]. Many efflux systems are involved in pumping out unwanted toxic substances. The MexCD-OprJ system is composed of three proteins: an efflux pump protein located in the cytoplasm, an OMP acting as a pore, and a protein located in the periplasmic space that connects the proteins located in the cytoplasm and the outer membrane [132].As a modification mechanism, *P. aeruginosa* alters the target by adding phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the lipid A of the bacterial lipopolysaccharides, thereby decreasing the affinity for the drug [133].

The last strategy examined is biofilm formation of *P. aeruginosa*. This complex architecture creates a protective environment for bacterial cells. The extracellular matrix forms a mechanical barrier that limits antibiotic diffusion within the biofilm and its access to microorganisms. Also, this matrix has an electrostatic charge that in some cases can bind to the antimicrobial and inactive it. Another mechanism described, is the reduction of the metabolism in some parts of the biofilms to avoid antimicrobials that targets metabolic processes like replication, transcription, translation, or cell wall synthesis [118,119,121].

In this context, the activation of efflux pumps by bacteria embedded in the extracellular matrix can also contribute to the inefficiency of antimicrobials by actively discharging them outside the biofilm structure before they reach their target. The bacterial density and spatial proximity promote horizontal gene transfer. The horizontal gene transfer could be 1000-fold more important in a bacterial community than between planktonic cells [134,135].

3.3 *Yersinia sp.*

Yersinia is a gram-negative, oxidase-negative, and facultatively anaerobic species. This bacterium is highly heterogeneous and can be divided into several genera, but only a few strains of *Yersinia* are associated with human disease [136]. *Yersinia* is an important human enteroinvasive pathogen with a grand distribution. In contrast to most other common bacterial enteropathogens, it can proliferate at different temperatures, from 4–37°C. This bacterium has the capacity to survive at low temperatures, for example temperatures found in refrigerators [137,138].

The virulence of pathogenic *Yersinia* is attributed to the presence of a highly conserved 70 kb virulence plasmid, termed plasmid for *Yersinia* virulence (pYV), which is absent in avirulent strains. This plasmid is essential for the bacterium to survive and multiply in lymphoid tissues[140,141].

As mentioned before, eleven species of *Yersinia* are known, but only three are considered pathogenic for humans: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica. Y. enterocolitica* and *Y. pseudotuberculosis* are gut pathogens (enteropathogens) causing gastroenteritis in humans and animals [136]. *Y. pestis* mostly resides in the blood (circulating or in fleas following blood meals) and lymph tissue, and it is the cause of the bubonic plague in humans and animals that is historically known as The Black Death [97,98]. *Yersinia enterocolitica* is associated with gastroenteritis, although in children we can find more severe clinical manifestations, for example, peritonitis. Due to the tropism in the lymphoid tissues and the fast spread of the bacterium via the bloodstream, generalized infections usually occur, resulting in meningitis, endocarditis, or aneurysm. As a result of the host's immune response, Y. *enterocolitica* can also induce secondary, postinfectious sequelae-like erythema nodosum and acute and chronic arthritis [141].

The use of antibiotics in *Yersinia* gastrointestinal infections is not recommended because these infections are self-limiting, and treatment is only recommended in immunocompromised patients who have an increased risk of developing bacteremia or even septicemia. The WHO has determined that the best treatment is with tetracycline, chloramphenicol, gentamicin, and cotrimoxazole. However, other compounds, such as ciprofloxacin and cefotaxime, are also considered since they have demonstrated excellent *in vitro* activity [138,140,141].

a.) Adhesion and intracellular survival of *Yersinia sp.*in host cells

It is well known that *Y. enterocolitica* can cause disease because of the virulence factors found in a 70 kb plasmid pYV. *Inv* and *Ail* genes encode a surface factor that enhances epithelial cell penetration. These genes have the capacity to bind to integrins of the β1 family of mammalian cell surfaces, allowing the entrance of bacteria by the zipper mechanism. In this case, the ability of the enteroinvasive bacteria to penetrate intestinal epithelial cells is the key to a successful invasive process [142].

After the invasion process, *Yersinia* is attacked by resident macrophages. In response, the bacteria produce and secrete protein effectors mediated by the T3SS. *YopH, YopT,* and *YopE* are three protein effectors that can disrupt the cytoskeletal assembly required for the phagocytosis process and remain extracellular in infected Peyer's patches and mesenteric lymph nodes. *Yersinia* is then disseminated locally and systemically, and, as a consequence, it can generate a systemic infection [143].

b.) Mechanisms of antibiotic resistance developed by *Yersinia sp.*

Y. enterocolitica has intrinsic resistance to β-lactamic antibiotics (e.g., ampicillin and the first-generation cephalosporins) due to the expression of two chromosomally encoded β-lactamase genes, namely blaA and blaB [144]. Two mechanisms of resistance to aminoglycosides have been characterized so far: the reduction of antimicrobial intracellular concentration via active efflux and the inactivation of the antibiotic by enzymatic modification, preventing the binding between the drug and the ribosomal RNA [145]. The resistance to quinolones is mediated by chromosome

and plasmid mechanisms. The chromosomal mutation affects the gene type-II topoisomerase protein targets (gyrA and gyrB for the DNA gyrase and parC and parE for the topoisomerase IV) and decreases the binding affinity toward the drug [146].

4. How improving knowledge of bacterial infect host mechanisms can help us to address resistance

Infectious diseases are annually the main cause of 15% of all deaths worldwide. As we mentioned before, MDR pathogens are rapidly spreading, and are exacerbated by the globalization. As such, the infectious disease field is set to face a series of challenges in the next decade that will require a revolution in our ability to rapidly understand, discover, and develop novel diagnostics. To address these challenges, infectious disease researchers are introducing systems biology approaches that allow high-throughput data and quantitative descriptions of molecular networks to fight against infection [147].

The objective of system biology is to study and model complex biological systems as a whole, consisting of the integration of information on single and multiple levels, from a focused to a general idea, such as genes, proteins, cells, tissues, whole organisms, or populations. All this information is unified by a mathematical model after the analysis of the high-throughput data. This new field of research has emerged in the 21st century, and the main idea is to join experimental data and computational analysis [147,148].

To have a complete view of system biology, there is a need to integrate all the experimental data with computational data. Once we obtain high-thought multi-omics data (such as genomics, transcriptomics, proteomics, etc.), this data is used to predict models of the networks and dynamic interactions of biological complexes. Recent technological advancements in high-throughput and quantitative measurements have allowed the application of systems approaches at the molecular level, which, until recently, was unthinkable. Each time the accuracy and sensitivity increase, the data become deeper and more precise. The models developed need a lot of testing and validation with the experimental data, but once the models are refined and corroborated, they can help us predict or identify therapeutic targets that can help diagnosis in the future [147-149].

A biological function cannot only be attributed to an individual molecule. Systems biology is the study of biological systems as a complex. Molecular biology has historically focused on molecules, such as studying a sequence of nucleotides or a protein, but the idea behind system biology is to combine all the information from single molecules into a system biology model. Thanks to system biology, we will be able to understand how these molecules and the interactions between them determine the function of complex machinery, both in isolation and when surrounded by other cells [150,151].

Many points of view need to be integrated and structured, such as gene regulatory and biochemical networks, physical structures, understanding dynamics of the system, both quantitative and qualitative analysis, construction of a model with powerful prediction capability, understanding control methods for the system, and understanding design methods of the system [150,151].

The resultant models are powerful tools for understanding the role of previously undescribed components and can help describe new relationships between components. For this reason, mathematical and computational models can help to diagnose and recommend therapy by focusing on biomarkers and drug targets if we think in terms of a whole organism.

4.1. Importance of system biology in infection

System biology is a powerful application for improving knowledge about infections. Applying system biology to infectious disease is complex because it involves two principal components: the host and the pathogen. The dual nature of these systems increases their complexity exponentially, and researchers have to consider how variations in either component may alter the dynamics and outcome of the overall relationship, as well as the interactions between each component [152,153].

The true complexity of the study of infection is to assimilate and integrate all the pathogen-host interactions and the response to each other that will define the infection process. We have to consider molecular, cellular, organismic, and even immune system responses at all levels. In the past, most studies focused on either the pathogen or the host. The union of mathematical modeling with bacteria-host data can be used to predict key network components or interactions; also, the integration of the perturbation of each and the effects of the changes of the network in the whole model need to be considered. In the past, research focused on either the pathogen or the host [152,153].

In both players, as a response to interaction, many signaling cascades are activated, which can likely change protein functions and, as a result, have different phenotypic responses. Pathogens try to adapt and modify themselves by hijacking the host for their own optimal replication and, at the same time, influence the host in response to this infection. Moreover, each disease is unique and depends on the species, pathogenic strain, environment, and immunological state of the host—resulting in a big challenge for the system biology of infectious diseases [154].

Recent technological advancements in high-throughput quantitative measurements have allowed the application of systems approaches at the molecular level. Several omics approaches, such as genomics, functional genomics, epigenomics, proteomics, and metabolomics, now allow for the identification and quantification of molecules in a system with increasing comprehensiveness, accuracy, and sensitivity. The development, application, and interpretation of these omics approaches, as well as the computational integration and modeling of the resulting datasets, evolve and grow with the technology [155,156].

All of these changes need to be considered at different molecular levels and can be quantified using omics, which involves computational analysis and modeling. Going beyond the analysis of dual transcriptomics simultaneously measures the gene expression of the host and the pathogen at the same time during an infection. So, using this data, we can understand the effect of each on the other after the processing and measurement of the transcript abundances. In fact, gene expression data of the host or pathogen during infection-related conditions or in vivo have strongly contributed to our knowledge about virulence factors, biomarkers, host immunity, and the dynamics of infection [156,157].

4.2 New technologies developed to study bacterial infection from a systems biology perspective

Access to large-scale omics datasets (genomics, transcriptomics, proteomics, etc.) has revolutionized biology and led to the emergence of systems approaches to advance our understanding of biological processes. With the decreasing time and cost to generate these datasets, omics data integration has created immense challenges for biologists, computational biologists, biostatisticians, and biomathematicians [156,157].

a.) Genomics

Genomics is the systematic study of the complete DNA sequences (genome) of organisms. Data obtained are compared with the reference genome available to analyze the differences and understand the DNA changes, because it has been demonstrated that small DNA changes can be responsible for differences found in the phenotype. Unfortunately, many diseases are caused by a single nucleotide mutation, and some DNA variation can be correlated with an increased risk of developing a particular disease, such as cancer [156-158].

Next-generation sequencing allows the determination of a sequence from the amplification of a single DNA fragment; however, its efficiency depends on higher throughput technology and a lower cost price per base [159]. As a comparison, Illumina has improved the reading length from the originally relatively short 50 bases to about 300 bases, also improving the sample preparation and reducing the sequencing cost, which was not expected just a few years ago.

In addition, other kinds of new techniques are appearing, such as chromatin immunoprecipitation sequencing (ChIP-seq) and single nucleotide polymorphisms (SNP)-ChIP. On the one hand, ChIP-seq is useful for analyzing protein interactors with DNA because it combines chromatin and immunoprecipitation with DNA sequencing to identify the binding sites of DNA-associated proteins [160]. On the other hand, the SNP-chip is a single nucleotide polymorphism and a DNA microarray that can detect polymorphism in a population. Single site variation in DNA is the most frequent type of variation in the genome. Curiously, around 335 million SNPs have been identified in the human genome [161].

b.) Transcriptomics

Transcriptomics is used to detect changes in RNA levels within the cell and to collect all the information at the regulatory level. The transcriptome of a cell is the pattern of gene expression at the level of gene transcription in a specific organism or under specific circumstances in specific cells [156,157,162].

Variations in mRNA levels provide an indirect quantification of the abundance of these proteins. This can be linked to several regulatory processes and pathways. Previously, RNA expression studies were performed using microarray chips, allowing for the study of thousands of genes. Nowadays, RNA-sequencing technologies can obtain the RNA expression of all the genes, together with more precise information on their abundance and the presence of splicing isoforms, which is interesting for understanding how infection is developed [162].

The recently developed dual-RNA sequencing technology provides a conceptually novel approach to the study of transcriptomes, allowing the host and pathogen transcriptomes to be analyzed in parallel [163]. Dual RNA-Seq is useful for investigating these infection dynamics. It is important to simultaneously capture the expressed genes of the pathogen-host, the molecular protein-protein interaction of bacterial infection processes, and the reciprocal host responses [164].

The benefit of such technology is the potential to monitor gene expression in both hosts and pathogens to a high level of sensitivity, accuracy, and depth. Most importantly, a dual approach will allow the monitoring of genes from both host and pathogen at different times after infection and throughout the infection process—that will give us the information from initial contact through to invasion and, finally, the manipulation of the host. It thus enables the temporal determination of responses and changes in the cellular networks in both organisms [165].

A complete dual RNA-Seq experiment comprises three steps: the first step is the obtention of total host-bacteria RNA extraction, then next-generation sequencing of total RNA, and finally, a bioinformatic processing and statistical analysis of the host-bacteria transcriptome. However, it is important to take into account that total RNA extraction is obtained from mixed prokaryotic and eukaryotic populations and that bacterial RNAs can constitute <1% of the total RNA in an infected cell. Moreover, up to 98% of the total RNA in an infected cell is eukaryotic ribosomal RNA, requiring rRNA depletion or mRNA enrichment. Additionally, traditional cell lysis techniques are often not suitable for both eukaryotic and prokaryotic organisms [166].

Most importantly, dual sequencing has opened up a new way of studying parallel access to the transcriptomes of hosts and pathogens, increasing knowledge of infections.

c.) Proteomics

Proteomics is the large-scale study of proteins in a particular biological setting; this biological approach provides the opportunity to detect post-translational modifications [156,157]. The analysis and quantification of expressed proteins are carried out using mass spectrometry-based methods [167,168].

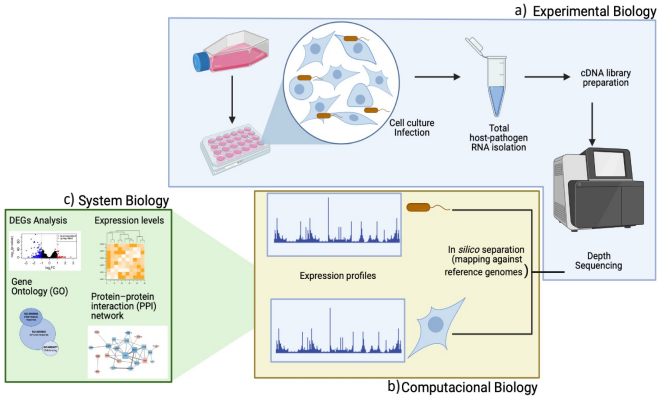

Figure 4. **A schematic representation of dual RNA sequencing and analysis. a)** Experimental biology undergoes the infection protocol and obtains the total RNA isolation. After obtaining good RNA quality, the cDNA library is prepared and then depth sequencing occurs. **b)** After the sequencing, we map the results with the reference genomes to obtain differentially expressed genes (DEGs). **c)** Once we obtain the DEGs, we can analyze the genes that are up-or down-regulated to know the expression levels. We can also do gene ontology analysis and obtain the protein-protein interaction network.

Mass spectrometry is an emerging method for analyzing and characterizing proteins. Currently, it is useful for the large-scale quantification of specific proteins in particular cell types under defined conditions. The rise of gel-free protein separation techniques, coupled with advances in mass spectrometry instrumentation sensitivity and automation, has provided a foundation for high-throughput approaches to the study of proteins [169,170].

Mass spectrometers separate compounds based on a the mass-to-charge ratio. To identify a sample, first needs to be ionized, then they must first be fragmented, formed and transferred into the gas phase before ions can be sampled and analyzed by a mass spectrometer. Then, each primary product is separated and quantified in the mass spectrometer according to its mass-to-charge ratio. The result, we obtain a mass spectrum of the molecules in the form of a plot when we can see the ion abundance versus mass-to-charge ratio. Ions provide us all the information concerning the nature and structure of their precursor molecules [170].

Mass spectrometry has contributed significantly to revealing protein-protein interactors and signaling networks and has helped to understand disease mechanisms. Moreover, it helps to identify unknown proteins of interest with the information obtained through the physical interaction with proteins of known function using affinity-purification mass spectrometry [171]. These techniques can be used to experimentally determine protein-protein interactions between hosts and pathogens during an infection, which could help to identify key participating proteins that can be used to design a new type of drug to prevent infections in the future [167-169].

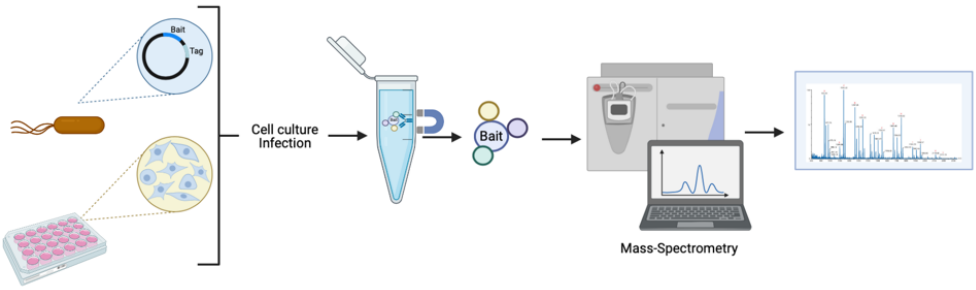

Figure 5. **A schematic representation to explain the experimental design of immunoprecipitation to analyze protein-protein interactors with the bait**. First, we start by tagging the bait. Once the mutant is validated, we perform an infection of cell culture. Eight hours post-infection, we extract the total protein, and we use the tag with agarose magnetic beads of the tag to separate our protein from the total. Once we do the cleanings and precipitate our bait with the interactors, we perform the mass-spectrometry analysis.

4.3 Multi-omics integration and mathematical modelling for understanding diseases

After the experimental design and data collection, the next major challenge is to use interpretable parameters with associated statistical measurements to analyze the data and assign significance. A mathematical model is a set of equations that describe how all parts of the system respond to system inputs, regulate each other, and control system output. These mechanisms are commonly represented in a network composed of nodes denoting molecules (for example, we can represent proteins, DNA, RNA, or metabolites) and edges representing an interaction between the connected nodes (protein-protein interactions, protein-DNA interactions) [172-174].

It is important to remember that the model is designed for the expressed purpose of visualizing and exploring data obtained in the multi-omics techniques. This representation should therefore be readily understandable, interpretable, and intuitive. In many circumstances, multiple types of representation, including networks, tables, heatmaps, and graphs, are required to effectively visualize different aspects of the model. Network models are designed to condense and represent complex datasets in simple ways, but a full understanding of the model often requires additional analysis of the network itself [172-174].

Many methods for network analysis have been developed, such as pathway or functional enrichment analysis, which are frequently used to reveal biological insights in large datasets or to guide functional follow-up studies. These methods are based on a comparison of the provided gene list to previously annotated curated lists of genes. A range of online tools is available to aid with this type of analysis, such as STRING, DAVID, gene set enrichment analysis, KEGG, and gene ontology [175-179]. These tools determine whether the genes or proteins identified in the network or subnetwork are enriched for any particular function, belong to any particular biological pathway, exist in any particular cellular complex, or share other commonalities.

Also, it is important to mention that the data need to be validated to establish confidence in a data set. For example, quantitative rtPCR can be used to confirm RNA sequencing data; reciprocal

immunoprecipitation or yeast two-hybrid screening or fluorescence resonance energy transfer can be used to confirm AP–MS data. A comparison with previously published data could also serve as a validation if the same parameters are monitored under the same conditions [180].

4.4 Applications of multi-omics and mathematical modelling

As we know, bacteria infect their hosts and hijack fundamental pathways that ensure their survival and proliferation. Accordingly, the capacity of infection of a pathogen can be related to its ability to interact with the host proteins. Once the high-through data are integrated with the correspondent bioinformatic analysis, we can predict the hubs in the host-pathogen interactome [177].

In addition, recognizing host proteins involved in pathogen interaction may pave the way for the rational design of new antibiotic molecules, the identification of disease biomarkers, and suitable therapeutic targets for high-throughput drug discovery. These efforts are currently progressing but are hindered by a lack of comprehensive data and appropriate bioinformatics solutions. Despite this, the unification of several individual omics enables us to link the design biomarker profiles to potential therapeutic targets. Therefore, it is a powerful way to obtain future successful drug discovery [181,182].

4.5 Specific examples of how bacteria use protein-protein interactions to hijack the host

As mentioned previously, *A. baumannii* is an emerging bacterium that causes many nosocomial infections, especially in intensive care units. Moreover, *A. baumannii* is resistant to almost all conventional antibiotics. For that reason, the development of a vaccine is one of the most promising and cost-effective strategies to prevent these infections. Chiang et al. identified potential protective vaccine candidates using reverse vaccinology, analyzing 14 online *Acinetobacter baumannii* available genome sequences, and found 2752 homologous core genes. This information was used to perform immunoproteomic experiments and predict the location of extracellular OMPs; 77 genes that could potentially be vaccine candidates were identified and selected for further studies. These antigens were found to be highly immunogenic and conferred partial protection (60%) in a pneumonia animal model [183].

Another representative example of the importance of system biology is the study by Kumar et al. regarding the antimicrobial resistance of *Pseudomonas aeruginosa*. In the study, they used a computational approach to understand resistance mechanisms that contribute to the bacteria becoming MDR. Thanks to the interaction network, 60 antimicrobial resistance genes were analyzed. Then, they performed an enrichment analysis, and as a result, they found that many genes were associated with efflux pump mechanisms, alginate biosynthesis, biofilm formation, and ampC beta-lactamase biosynthesis. These genes can be used as potential drug targets for developing new drugs against MDR *Pseudomonas* infections. [184]

Schmuh et *al.* carried out a comparative high-resolution transcriptome analysis of different *Y. enterocolitca* strains. The RNA sequencing revealed 1,076 identified strain-specific sRNAs that could contribute to differential regulation among the phylogroups. They report new insights into identifying the different phylogroups, taking into account the specific transcriptome organization of the groups [185].

5. How we propose to understand infection biologically and solve antibiotic resistance from the point of view of protein-protein interaction

The systems biology of microbial infections aims to describe and analyze the confrontation of the host with bacterial and fungal pathogens. This comprises experimental studies that provide spatio-temporal data from monitoring the response of the host and pathogenic cells to perturbations or when interacting with each other, as well as the integrative analysis of genome-wide data from both the host and the pathogen.

Accordingly, with the results obtained and after the integration of all the data of omics, the studied protein-protein interactions may help us to understand how the infection develops. Systems biological studies comprise both experimental and theoretical approaches. The experimental studies may be dedicated to revealing the relevance of certain genes or proteins in the above-mentioned processes on the side of the pathogen or the host by applying functional and biochemical analyses based on knock-out mutants and knock-down experiments.

If we can follow the interactions at different points after infection, we can determine the differentially expressed genes at the different points of the infection. In perspective, the host-pathogen interaction should be described by a combination of spatio-temporal models with interacting molecular networks of the host and the pathogen.

Gene expression is mainly regulated by transcription factors and co-factors, as well as by post-transcriptional modification and mRNA and protein degradation. For this reason, if we compare the number of readings obtained with the control, we can determine the genes that are upregulated or downregulated during infection.

Then, we can perform gene ontology analysis to understand the functions of the different groups of affected genes, which will give us an idea of the affected pathways. After all the integration and all the analysis, we can construct a protein-protein interaction network where we can identify key proteins or the hubs.

The aim is to unravel the main mechanisms of pathogenicity, to identify diagnostic biomarkers and potential drug targets, and to explore novel strategies for personalized therapy by computer simulations. At that point, we proposed studying these hubs as target proteins for the development of new antibiotics. There is an urgent need to advance and obtain antibiotics. Also is important to mention that it is like a targeted therapy that maybe there will not be as many side effects.

AIMS OF THE THESIS

The overall objective of the present thesis is to study the mechanisms of bacterial infection, more specifically on protein-protein interactions, which are established between the host and the pathogen during an infection process. More specifically, it consists of an exhaustive study of the transcriptional changes, both of the pathogenic bacterium and the host during the infection process. Studying reciprocal genetic changes during an infection can help us understand host-pathogen interactions and their importance. Specifically, the particular aims of this work are:

1. To establish the relationship between host-pathogen interactivity connectivity and pathogen fitness during an infection.

2. To determinate the transcriptional changes in epithelial cells during an *Acinetobacter baumannii* infection

3. To characterize the formation of biofilms to identify relevant protein-protein interactions in *Pseudomona aeruginosa* infection in lung epithelial cells derived from healthy or CF-affected patients.

4. To identify and characterize new virulence factors in bacteria

RESULTS

- **CHAPTER I:** Centrality in the host–pathogen interactome is associated with pathogen fitness during infection

- **CHAPTER II:** Time-Resolved Transcriptional Profiling of Epithelial Cells Infected by Intracellular *Acinetobacter baumannii*

Papers I and II are included as main papers of the thesis.

CHAPTER I: Centrality in the host–pathogen interactome is associated with pathogen fitness during infection

Crua Asensio, N., Muñoz Giner, E., de Groot, N. et al. Centrality in the host–pathogen interactome is associated with pathogen fitness during infection. Nat Commun 8, 14092 (2017). https://doi.org/10.1038/ncomms14092

CHAPTER II: Time-Resolved Transcriptional Profiling of Epithelial Cells Infected by Intracellular *Acinetobacter baumannii*

Crua Asensio, N., Macho Rendón, J., & Torrent Burgas, M. (2021). Time-Resolved Transcriptional Profiling of Epithelial Cells Infected by Intracellular Acinetobacter baumannii. Microorganisms, 9(2), 354. https://doi.org/10.3390/microorganisms9020354

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

CHAPTER I: Centrality in the host–pathogen interactome is associated with pathogen fitness during infection

Technological advances are continuously advance scientific knowledge, by improving the accuracy and sensitivity of more traditional methods or allowing to undertake new approaches to relevant questions. Recently, the view of biological complexes, using a system biology perspective has changed the way we understand biology. The study of the whole systems using high-through data and integrated computational analysis has let us obtain more detailed information on the biological processes. Focusing on infection, the possibility of studying at the same time the host and pathogen interrelations has open many doors in research.

One open field is the study of the protein-protein interaction during an infection to figure out the effects of pathogen survival. In this context, when we compared the *Yersinia pestis-Homo sapiens* interactome with *Yersinia pestis* interactome, both in *vitro* and in *vivo*, we observed little correlation between infection fitness in both cases. By representing the percentage of essential protein versus the number of edges linked to this protein (degree), we concluded that the infection fitness contribution of individual genes is correlated to the interaction degree only in the case of the host-pathogen interactome but not with the *Y.pestis* interactome alone.

These results endorse our hypothesis that, during infection, there is a positive correlation between the number of interactions with the host and the contribution of a particular protein to infection. Moreover, the same results were confirmed with other bacteria such as *Acinetobacter baumannii* and *Salmonella enterica*. These results are in good agreement with the centrality-lethality rule proposed by Jeong et al. in 2001, "The most highly connected proteins in the cell are the most important for its survival". According to the centrality-lethality rule, that hub proteins in a network tend to correspond to proteins that are more essential and robust.

Following this observation, we plotted the fitness, considered as the effect of deleting a certain protein in infection, both *in vivo* (animal models) and *in vitro* (test tube). The result was a surprising non-existent to negative correlation, indicating relevant deletions *in vivo* does not have, most times, a significant affect in *vitro,* and vice versa. Using this analysis, proteins could be classified in three different groups: proteins that have a high effect only when deleted *in vivo*, proteins that have a high effect only when deleted *in vivo* or proteins have with a neutral effect in both cases, with most of the proteins belonging to the latter group, as expected. This result led us to investigate whether such behavior could be explained by the centrality-lethality rule.

In fact, we observed that proteins that compromise pathogen infection, represent hubs in the host-pathogen interactome while proteins that compromise pathogen growth were hubs in the pathogen interactome. Gene ontology (GO) enrichment calculations showed that these hubs in the host-pathogen interactome are involved in relevant functions during infection, including the immune response or regulation of membrane vesicle formation.

Finally, we evaluated how changes in the host-pathogen network can affect infection, by targeting individual nodes in the host-pathogen interactome. To analyze the effect of deleting a particular node in the interactome, we removed that node and calculated the global effect in the network. To measure such global effect, we used a metric called global topological efficiency (GE), defined as the average of the inverse of the shortest path between two nodes in the network, and it represents the efficiency in the communication between all the nodes in the network. We expected that an increasing number of disrupted nodes would induce a GE reduction. To obtain representative results, we measured the decrease in GE after consecutive node removal in the network using three different approaches: a random attack, a pathogen-directed attack, and a centrality-based attack. From these calculations, we observed that a centrality-based attack was the most effective disrupting the global topological efficiency. However, the pathogen-directed attack efficiency in disrupting the network was in between the random and centrality-based strategies, suggesting that it is in the pathogen's best interest not to destroy the host network of contacts, but to hijack only the most relevant pathways to ensure its propagation without triggering an irreversible damage to the cell.

In summary, the significant correlation observed between connectivity and fitness allowed us to postulate that highly connected pathogen proteins in the host-pathogen interactome have a high impact on the fitness of the organism during infection, following a new centrality-lethality rule for multiorganism networks. We also suggest that future investigations of the protein-protein interactions at different time points during infection can lead us to know the time-dependent interactions and how these changes affect infection. This kinetic view of the host-pathogen protein-protein interaction maps can become a revolutionary way to design and develop antimicrobials.

CHAPTER II: Time-Resolved Transcriptional Profiling of Epithelial Cells Infected by Intracellular *Acinetobacter baumannii*

Acinetobacter baumannii is emerging as one important drug-resistant bacteria and is responsible for many hospital-and community-acquired infections. Some strains are resistant even to carbapenems and polymyxins. So, at this point there is an urgent need to study *A.baumannii* infection: how the bacteria can infect and the host response to *A.baumannii* infection to understanding the pathogen effects in host cells that can be used in the future to develop new therapeutic tools. The adherence of *A.baumannii* to infect cells has been described much time, this bacteria can infect host cells via zipper-like mechanisms, and OmpA protein is considered the protein is considered to be responsible for directing the entry of the bacterium but how these bacteria survive inside the host cell is not clear.

Once explored the situation from multiple perspectives, we validated by different experiments the capacity of *A. baumannii* to survive and replicate inside epithelial cells. Although these findings reflect a low cellular invasion of *A. baumannii* in epithelial cells, our results were also backed up by several studies in the literature. A relatively low adherence and invasion of *A. baumannii* in epithelial cells may contribute to a low virulence of this opportunistic pathogen, which particularly infects critically ill or severely wounded patients. This information can give us an idea of the persistence over time of *A. baumannii*, helping the bacteria to obtain antimicrobial resistance giving and limiting further therapeutic options.

In this context, we investigated the effects of the *A.baumannii* infection in the host cells using dual RNA sequencing. RNA analysis at different times post-infection allowed us to obtain a detailed picture of the infection impact in host cells over time. In agreement with the previous results, we found only two genes differently expressed at 2 hours post-infection after the RNA-sequencing analysis. Also, as expected, an increasing number of differentially expressed genes (DEGs) was found at the next two times post-infection, as we can see in figure 6. This information reinforces then idea of a time-dependent rearrangement of the host network.

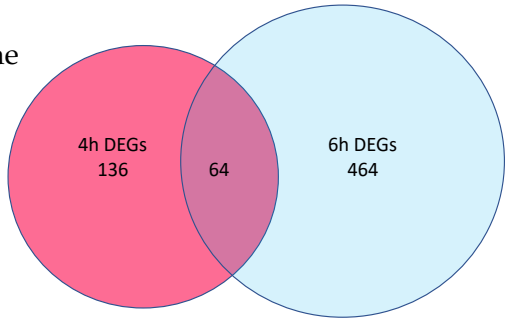

Figure 6.- **Venn diagram of differentially expressed genes of an infection of *A.baumannii*.** In pink we can see the number of DEGs found at 4h and in blue at 6 h post-infection. In the middle, the DEGs that are both affected at 4 and 6 h post-infection.

In our model, almost all DEGs appeared as downregulated genes. A detailed analysis of our results suggests that the bacteria have the capacity to subvert the host signaling networks in a time-depending manner, by targeting the host's immune response and the production of the cytokines in specifically on early time points. This means that, over time, *A. baumannii* can hijack the immune system and reduce the cytokine cascade to prevent the activation of innate immunity. Later, at 6 hours post infection, there is a significant downregulation of the inflammatory response, TNF, and MAPK signaling pathways. Hence, the bacterium can inhibit the pro-

inflammatory pathways, which are responsible for activating the adaptive immune response, allowing *A. baumannii* to evade the host cell defense, promoting its survival inside the cell.

Form the results, host cells also try to counteract bacterial infection, by increasing the expression of the HLA class II histocompatibility antigen, particularly at 4 hours post-infection. The response mounted by the host makes perfect sense, as this antigen has the mission to activate professional and non-professional antigen-presenting cells, allowing the activation of the adaptative response. It is also important to highlight that, because these antigens are obtained from an endocytosed particle in the lysosomes, the peptide fragments are presented to the cell surface. Following this idea, we can assume that *A. baumannii* is engulfed and digested in lysosomes, which is compatible with the idea proposed by Raquel Parra-Millán et al.

With the ability to reside inside lysosomes, bacteria can better resist antimicrobials, facilitating the dissemination of the bacteria over the host. Here, we would like to point out that several proteins such as the heat shock 70 kDa protein 6 (HSPA6), the RAS-related protein Rab-3A (RAB3A), and the Thioredoxin-interacting protein (TXNIP), all related to membrane trafficking, are upregulated 6 hours post-infection. This fact makes us think that *A.baumanni* keeps them upregulated for its own interest, this cell mechanism is important for the bacteria to survive and spreading and consequently, the bacteria is maintaining upregulated until the bacterium can leave the host cell.

Also, we found a clear activation of several pathways related to cell death and apoptosis, such as the activation of DNA damage-inducible transcript 4 protein (DDIT4) and other proteins involved in generating reactive oxygen species (ROS). Activating apoptosis and stress response is a well-defined strategy used by cells to avoid the bacteria dissemination between host cells. Different articles also observed that high levels of ROS and DDIT4 can inhibit bacterial grown.

Another hypothesis that we came up with is to identify the possible post-transcriptional regulation genes, to see changes in the regulation of host cells in *A.baumannii* infection. We did not see significant differences in the upregulated, but in the downregulated transcripts we find longer transcripts and 5' -3' untranslated region (URTs). These studies allow us to assume that longer URT has more ribosome binding sites and this may say that there is a faster transcription, in this group we find several transcription factors, including NFKB1, DBNT1, and CCBP, related to inflammation and zinc finger that regulate methylation.

Further, using the Spring data base we could connect the proteins encoded in the DEGs. Determining the protein-protein network helps us to reveal the genes that are highly connected. For example, in our network DUSP5, TNF, CTGF, and CXCL can be considered hubs, because they are connecting almost all the DEGs. These results suggest that transcriptional changes are also coordinated with protein-protein interactions. In the future, this network can be used to design small molecules or peptides to inhibit these interactions and may probably avoid *A. baumannii* infection.

In summary, we have shown that *A. baumannii* can infect and replicate inside epithelial cells and hijack the host cell machinery to its own benefit, as observed in other well characterized intracellular bacteria such as *Yersinia enterocolitica*. These results show the that is important to change the current classification of *A.baumannii* being only an extracellular bacteria. Also, we have seen how these bacteria can avoid host defense and promote its replication.

CONCLUDING REMARKS

As a general conclusion, this thesis aims to enlighten new perspectives in infectious disease. By studying protein-protein interactions during infection we can open new fields of research and can help to infections at different levels of complexity.

The relation observed between pathogen fitness and the centrality in host-pathogen interactome opens a new paradigm to understand infection and may pave the way to the discovery of new antimicrobials. Also, our results showed that current binary classifications of pathogens between intracellular and extracellular are not clear. *A. baumannii,* as an extracellular bacterium, also display a rich intracellular lifestyle and can modulate the host response to ensure its survival. These approaches may inspire the development of new treatments for *A.baumannii* infections. Although much more research is required, this thesis has provided new points of view that will open new lines of research and perhaps inspiring the development of new antimicrobial drugs.

CONCLUSIONS

Chapter I: Centrality in the host-pathogen interactome is associated with pathogen fitness during infection

- The number of interactions and the percentage of essential proteins can be correlated in the *Y.pestis-Homo sapiens* interactome during an infection but not in the *Y.pestis* interactome.

- Centrality-lethality rule can be applied in the proteome of a host-pathogen in *vivo*.

- The fitness has a negative correlation between in vivo and in vitro assays.

- The functionality of the protein-protein interactors does not depend only on the pathogen proteins, it needs to consider the interacting partners.

- Global topological efficiency is highly affected by the hub proteins in *vivo* assays.

- The key proteins in the host-pathogen interactome are connected with pathogen fitness during an infection.

Chapter II: Time-Resolved Transcriptional Profiling of Epithelial Cells Infected by Intracellular *Acinetobacter baumannii*

- *A.baumannii* can survive and replicate in host epithelial cells by controlling host response.

- Intracellular lifestyles give advantages to antimicrobial resistance.

- Protein-protein interactions network between host-pathogen can mark hub proteins and can be used as a target for developing new antimicrobials.

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