1. Introduction

1.1 Antimicrobial resistance

Antimicrobial resistance (AMR) is a growing concern for global human health, as it poses a significant challenge to the treatment of infectious diseases. The misuse of antibiotics is one of the major contributing factors to the emergence of AMR. Antibiotic resistance genes (ARGs) can be detected in a wide range of environments, including natural, engineered, and clinical settings (Figure 1). [1][2].

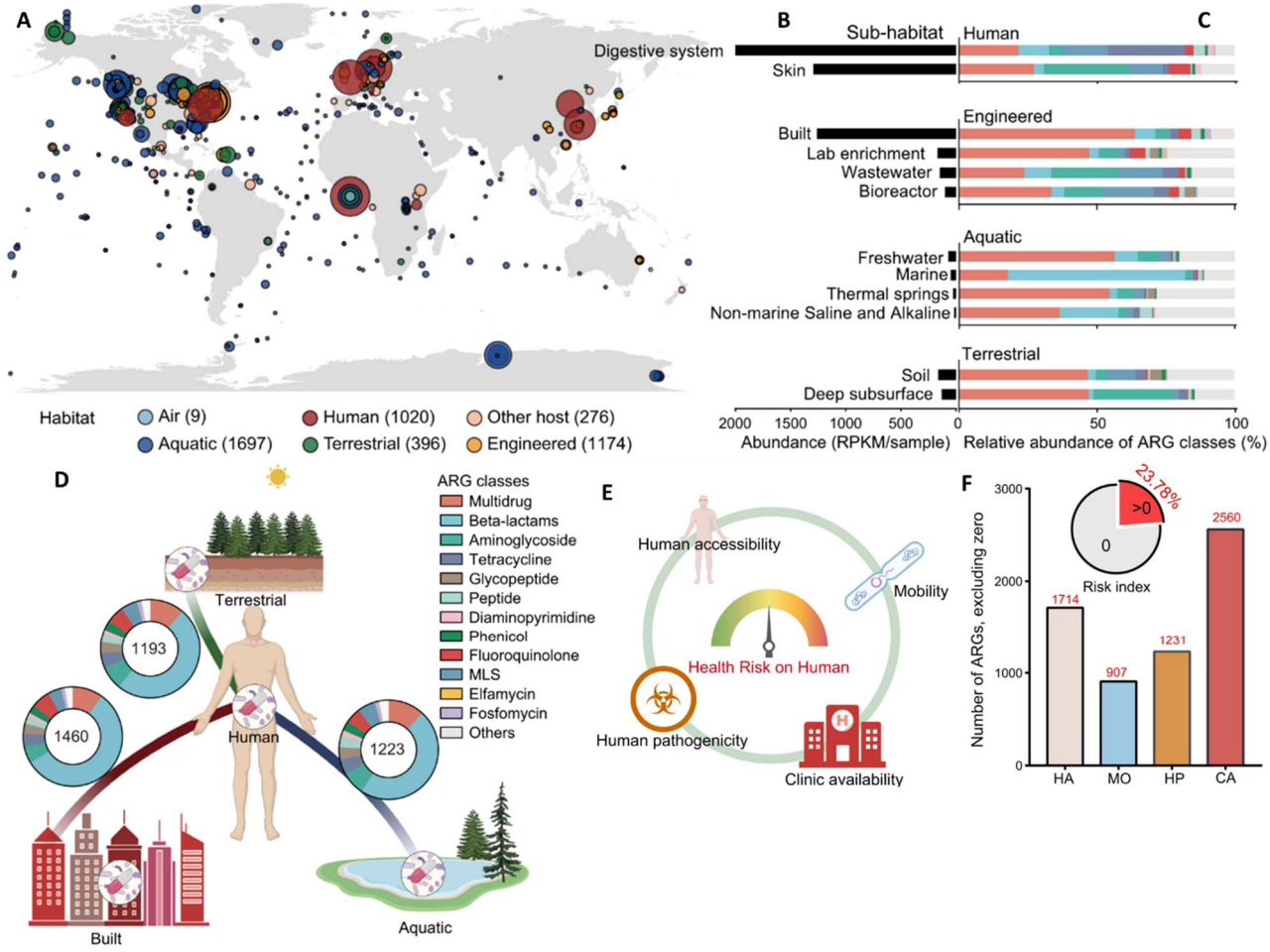

Figure 1: Worldwide representation of antibiotic resistance genes problematic. [1] **A.** Geographic distribution of samples with ARG abundance in various habitats. Each point indicates one sampling location, rounded to the nearest degree, with point size reflecting the number of samples, and point color indicating the habitat. Samples with the unclassified location was shown as longitude = 0 and latitude = 0. **B.** Abundance of ARGs in each sub-habitat. Digestive system of human, mainly including the fecal samples, had the highest abundances of ARGs. **C.** Composition of antibiotic resistome in each sub-habitat. Only sub-habitats containing at least 20 samples are shown. **D.** ARGs shared between the human-associated and three main habitats. Number in the circles represents the number of shared ARGs. **E.** Health risk of human for each ARG with four indicators, including human accessibility (HA), mobility (MO), human pathogenicity (HP), and clinical availability (CA). **F.** Number of ARGs after excluding the zero value in each indicator for risk index calculation. Only 23.78% of all evaluated ARGs exhibited risk index (RI) > 0. RI of most ARGs was zero because of the strict formula we used.

The rise of multidrug resistance (MDR) in combination with the limited development of new treatments poses a significant threat to the effectiveness of our healthcare system. Despite this, AMR remains a silent pandemic that has not subsided. To address this issue, research must focus on understanding the mechanisms of resistance in resistant bacteria and identifying strategies to prevent the emergence of antibiotic-resistant "superbugs." [3].

1.2 Origin of resistance

One of the mechanisms by which bacteria acquire resistance is through point mutations. These mutations can occur naturally and can give bacteria a small selective advantage, allowing them to survive in the presence of an antibiotic [4]. Another way bacteria can acquire resistance is by the transfer of plasmids containing antibiotic resistance genes through horizontal gene transfer. This can happen between different bacterial species and can lead to the rapid spread of resistance. The ability of bacteria to transfer resistance genes through plasmids and the emergence of resistance through point mutations are major factors in the development and spread of antibiotic resistance [5].

Antimicrobial resistance is a phenomenon that has been observed for centuries, with the earliest known reports dating back to the mid-20th century. However, the widespread use of antibiotics in the 21st century has accelerated the emergence and spread of AMR. The first discovery of antibiotic resistance was in the 1940s, when penicillin-resistant strains of *Staphylococcus aureus* were identified [6]. Since then, resistance to a wide range of antibiotics has been observed, including tetracycline in the 1950s, methicillin in the 1960s, and vancomycin in the 1980s. The emergence and spread of MDR bacteria, including methicillin-resistant *Staphylococcus Aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE), has highlighted the ongoing co-evolution between antibiotics and resistance mechanisms. The increasing prevalence of AMR is a major public health concern, as it undermines the effectiveness of antibiotics and increases the risk of treatment failure.

1.3 Mechanism of resistance

Antibiotics can be classified into 15 major classes and have different mechanisms of action against bacteria (Annex1). Most antibiotics interact directly with enzymatic processes within bacteria, targeting cell wall synthesis (beta-lactams), ribosomes (chloramphenicol), nucleic acid machinery (fluoroquinolones), or bacterial membranes (sulfonamides) [7,8]. Resistance to antibiotics can occur through various mechanisms, which can be broadly categorized into two main effects, prevent the antibiotic from reaching its target or modify the target itself (Figure 2).

Preventing the antibiotic from reaching the target can be achieved via:

- Efflux pumps: bacteria possess membrane or cell wall pumps known as efflux pumps, which actively transport antibiotics out of the bacterial cell. These pumps are common in bacteria and can transport various compounds. Increased production of these pumps due to mutations in bacterial DNA contributes to antibiotic resistance. (QacA transporter in *S. Aureus*)
- Decreased membrane permeability: changes in the bacterial membrane protein composition can reduce the passage of antibiotics into the bacterial cell, thereby decreasing their effectiveness. (Decreased expression of the outer membrane porin OmpF in *E. Coli* [9])
- Enzymatic degradation: bacteria produce enzymes, such as β-lactamase, that can degrade antibiotics. β-lactamase, for example, destroys the active component (β-lactam ring) of penicillin. Some bacteria, known as “Extended-spectrum beta-lactamases” (ESBLs)-producing bacteria, have developed ESBLs that can degrade a wide range of β-lactam antibiotics, including last-resort drugs.
- Modification of antibiotics: bacteria can produce enzymes capable of adding chemical groups to antibiotics. This modification prevents the antibiotic from binding to its target within the bacterial cell, leading to resistance. (Lincosamide antibiotics can be modified by nucleotidyltransferases like lnu(A) in *Staphylococcus* species [10])

Modifying the target of the antibiotic can be achieved via:

- Camouflage of the target: bacteria can undergo changes in the composition or structure of the antibiotic target through mutations in bacterial DNA. This alteration prevents the antibiotic from interacting effectively with the target. Additionally, bacteria can add chemical groups to the target structure, providing a shield against the antibiotic. (Methyl transferase enzyme, ErmC in several strains of pathogenic bacteria [11])
- Express alternative proteins: some bacteria can produce alternative proteins that can substitute for the ones inhibited by the antibiotic. For instance, *Staphylococcus aureus* can acquire the resistance gene *mec*A and produce a different penicillin-binding protein. These proteins are essential for the bacterial cell wall synthesis and are targeted by β-lactam antibiotics. The different penicillin-binding protein exhibits low affinity to β-lactam antibiotics, rendering the bacteria resistant to the drugs and enabling their survival. This resistance mechanism is fundamental in MRSA [12].
- Reprogram the target: bacteria can generate a different variant of a required structure. For example, VRE bacteria develop a distinct cell wall compared to susceptible bacteria. This altered cell wall diminishes the antibiotic's ability to interact effectively, contributing to resistance against vancomycin [13].

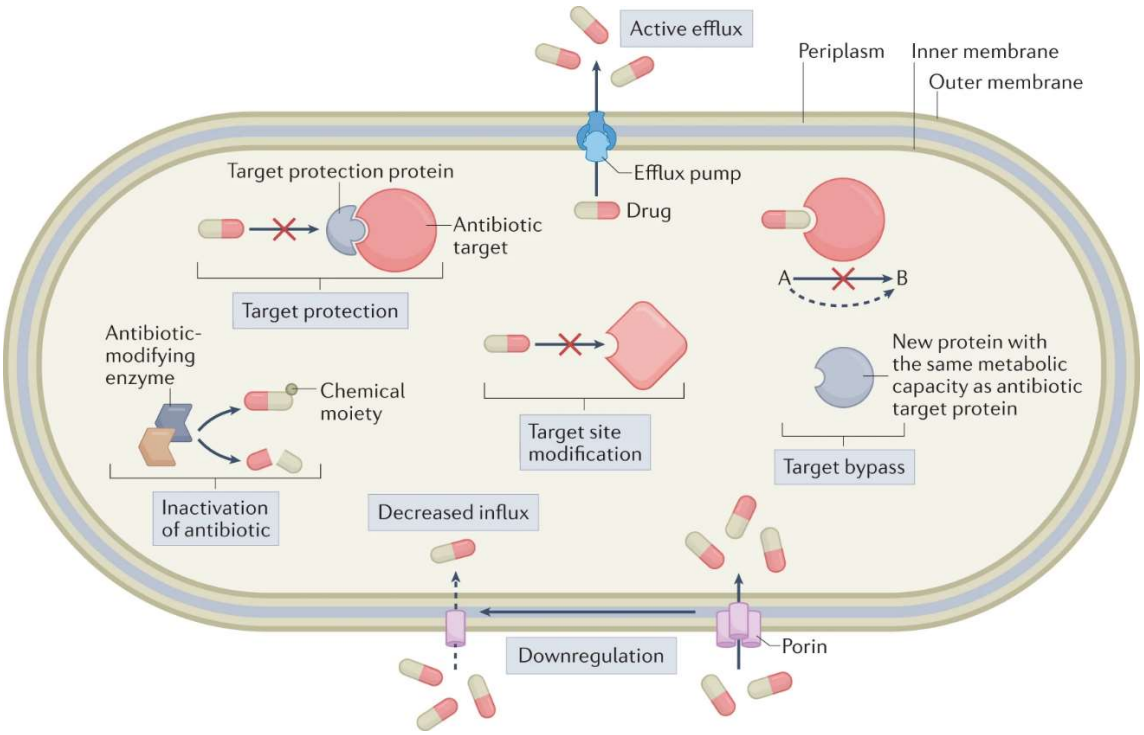

Figure 2: Overview of the molecular mechanisms of antibiotic resistance. Antibiotic inactivation is achieved through multiple enzymatic and non-enzymatic mechanisms. Enzymatic degradation involves the hydrolysis of the antibiotic's functional group, leading to its ineffectiveness. Antibiotic-modifying enzymes transfer various chemical groups to the antibiotic, preventing its binding to the target. Target site alteration involves modifying the antibiotic target to reduce its affinity for the antibiotic, which can occur through mutations in the target gene or enzymatic modification of the binding site. Target bypass occurs when a different protein takes on the role of the antibiotic target, bypassing its inhibition and rendering the original target redundant, thereby making the antibiotic ineffective. Decreased influx of antibiotics into bacterial cells can result from alterations in the membrane structure, such as the downregulation of porins. Porins are transmembrane proteins that facilitate the passive transport of various compounds, including antibiotics. Active efflux of antibiotics is facilitated by transmembrane efflux pumps, which actively export antibiotics out of bacterial cells, thereby reducing their intracellular concentration. Target protection involves the physical association of a protective protein with the antibiotic target, relieving it from the inhibitory effects of the antibiotic. These mechanisms collectively contribute to the inactivation and resistance of antibiotics by bacteria. (From [14])

1.4 Multidrug transporter

Efflux pumps play a prominent role in MDR due to their high efficiency and polyspecificity, making them crucial in the defense mechanisms of bacteria. These pumps are all located within the membrane or possess a transmembrane domain, and they are found in both gram-positive and gram-negative bacteria.

Multidrug transporters can be categorized into many different families (Figure 3). In more recent years, seven families have been identified [14] which can be further grouped into five superfamily categories. Each characterized by specific criteria such as composition, number of transmembrane helices (TM), energy sources, and substrate recognition [15]. The five families are as follows:

- ABC (ATP-binding cassette) superfamily: This family of transporters is characterized by the use of ATP as an energy source to drive the efflux of drugs. One example of a bacterial ABC transporter is Sav1866 from *S.aureus* . [16]
- MFS (major facilitator superfamily): The MFS family comprises transporters that facilitate the movement of drugs across the membrane by utilizing a proton gradient or ion antiport. An example of an MFS transporter is the NorA protein found in *S. aureus.* [17]
- MATE (multidrug and toxic compound extrusion) family: The MATE family is a large family of secondary active transporters. MATE is involved in the transport of various xenobiotics and metabolites across cellular and organellar membranes using electrochemical potential of ions or protons. An example of a MATE is the NorM protein found in *Vibrio cholerae*. [18]
- SMR (small multidrug resistance) family: As their name implies, these small (~12 kDa) integral inner membrane proteins range from 100 to 140 amino acids in length and confer resistance to a variety of quaternary ammonium compounds (QAC) in addition to other lipophilic cations. They are also proton-dependent multidrug efflux systems. An example of an SMR is EmrE found in *E. coli*.[19]
- RND (resistance-nodulation-division) superfamily: The protein from the RND family relies on the proton motive force and usually form tripartite efflux pumps complex with other proteins. An example of an RND inner membrane protein is the AcrB protein found in *E. coli*. [20]

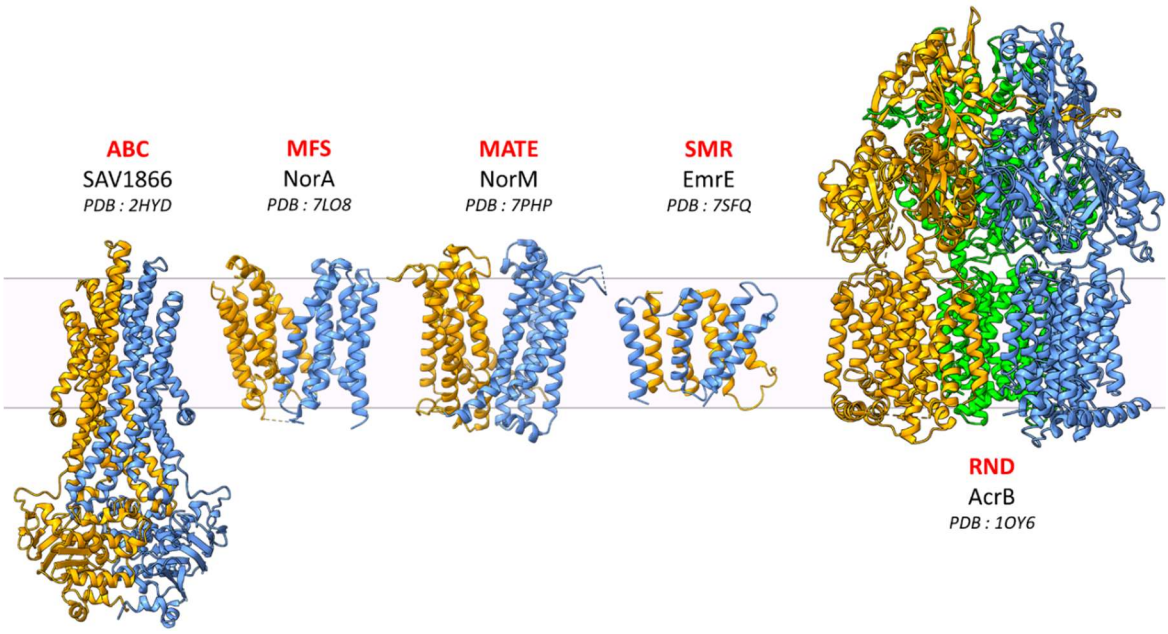

Figure 3: Structural representations of MDR transporter families. The N lobe is depicted in blue, while the C-lobe is shown in orange. In the case of protein complexes, each monomer is represented in a distinct color, including orange, blue, or green. An example for each family is represented: SAV1866 for the ABC family (PDB:2HYD), NorA for the MFS (PDB:7LO8), NorM for the MATE family (PDB:7PHP), EmrE for the SMR family (PDB:7SFQ) and AcrB for the RND family (PDB:1OY6).

1.5 Major Facilitator Superfamily

The Major Facilitator Superfamily (MFS) is the largest and most diverse superfamily of secondary active transporters, accounting for approximately 25% of all known membrane transport proteins in prokaryotes. It consists of 58 distinct families, with over 15,000 sequenced members identified so far [21]. The MFS is widely present in all kingdoms of life and plays crucial roles in growth, metabolism, and cellular homeostasis. Within the MFS, various subfamilies have evolved to perform specific functions in their respective domains.

MFS transporters generally comprise 400 to 600 amino acid residues and exhibit low sequence conservation of 12-18%. The canonical most represented MFS-fold has 12 transmembrane (TM) segments organized from two 6-TM bundles connected by a long and flexible intracellular loop. While they may be small compared to other multidrug transporters families, they possess an impressive diversity of substrates. This diversity encompasses sugars, polyols, drugs, neurotransmitters, amino acids, peptides, lipids, organic and inorganic ions, vitamins, nucleobases, nucleosides, and nucleotides [22].

MFS transporters exhibit three different mechanisms: uniporters, symporters, and antiporters. Uniporters transport a single type of substrate and rely solely on the substrate gradient for energy. Symporters simultaneously translocate two or more substrates in the same direction, utilizing the electrochemical gradient of one ligand as the driving force. Antiporters transport multiple substrates in opposite directions across the membrane. Secondary-active MFS symporter and antiporter can either utilize a proton or a sodium-motive force. In the case of the proton motive force (PMF) also known as the proton electrochemical gradient, the specific characteristics of the bacterial inner membrane, such as the negative inside membrane potential ($\Delta\psi$) and alkaline inside pH gradient (ΔpH), play a crucial role in governing the movement of various solutes, which accounts for the versatility of efflux pumps in handling electrically distinct substrates. The substrate binding pockets in these transporters are typically spacious and flexible, enabling the binding of substrates through nonspecific electrostatic and hydrophobic interactions [23].

Regardless of the energy source, the fundamental principle underlying the transport mechanism involves the alternating exposure of the substrate-binding site to only one side of the membrane at any given time. This mode of action is known as the "alternating-access model" (Figure 4A), and previous investigations have revealed that the alternating-access mechanism can generally be described by three distinct types of models: the "rocker-switch," the "rocking-bundle," or the "elevator" mechanism. Over the past decade, a combination of transporter structures in various functional states, coupled with biochemical and biophysical analyses, as well as in silico molecular dynamics (MD) simulations, has provided deeper insights into the specific details of their transport mechanisms.

1.5.1 MFS Multidrug Resistance Transporter

In the case of this study, we will focus on the multidrug transporter represented among microbial genomes. The most widespread and understood microbial MFS-MDR transport systems belong to the 12-TM drug:H+ antiporter 1 (DHA1) and 14-TM drug:H+ antiporter 2 (DHA2) families [24]. Among DHA1 members, crystal structures are available for *E. coli* transporters MdfA (PDB:4ZOW), EmrD (PDB:2GFP) and YajR (PDB:3WDO) in different conformational states [25,26,27]. Extensive studies on MdfA and LmrP from *L. lactis* revealed diverse aspects of their functional properties involving substrate promiscuity, H+:drug stoichiometry and sites for substrate recognition [28,29]. In contrast, with only one available structure of NorC [30], the DHA2 family of antiporters have not been characterized extensively.

The major conformations observed for the MFS-MDR DHA1 and 2 proteins all lead to the Rocker-switch model. This model was further improved and always involve the transition of the protein between three different states, the outward open (OO), the inward open (IO) and the occluded (Figure 4B). An extension of this model was proposed by our lab and suggest an alternative mechanism for substrate access to the binding pocket (Figure 4C). This new model considers data obtained on LmrP from various experimental techniques such as Double Electron-Electron Resonance (DEER), Single molecule Förster resonance energy transfer (smFRET), and more recently crystallographic studies.

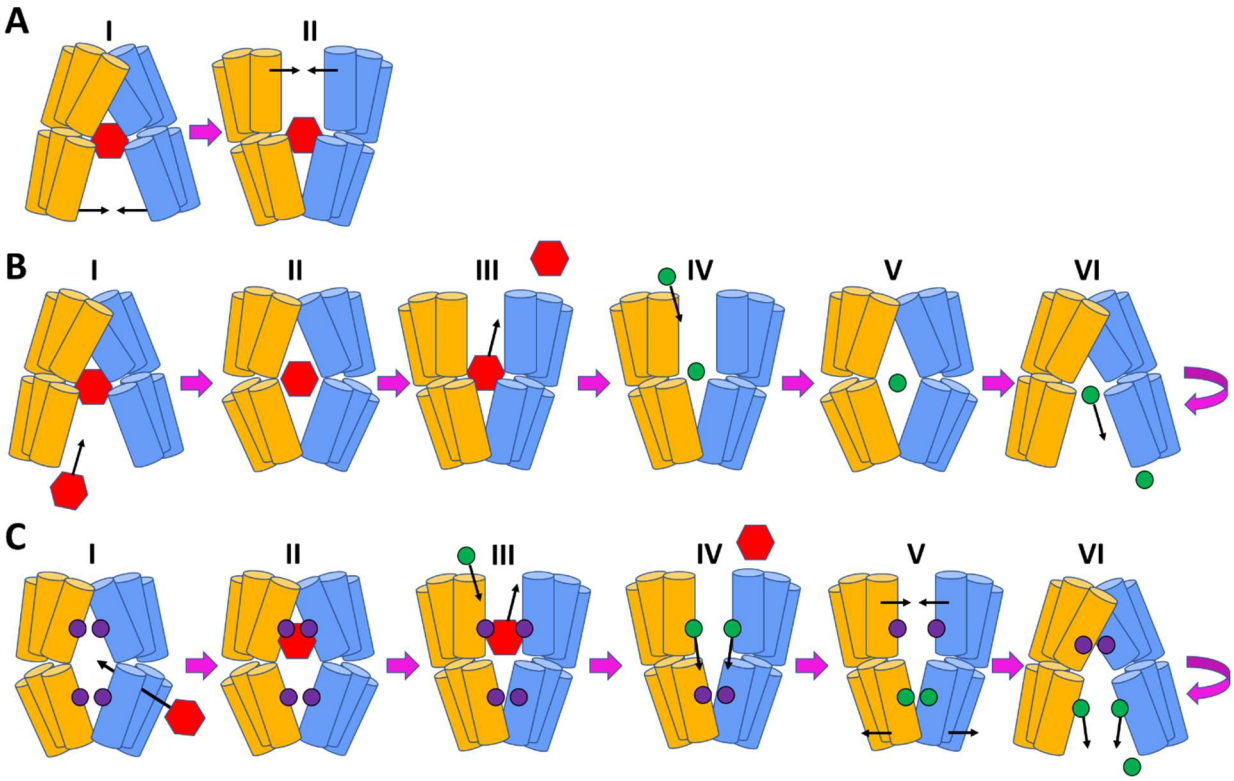

Figure 4: Proposed model of transport cycles of MFS. [22] **A:** In the rocker-switch mechanism, the structurally similar bundles (N lobe (light blue) and C lobe (light orange)) rearrange symmetrically around the centrally located substrate-binding site (substrate shown as red hexagon) to alternate access to the other side of the membrane. **I.** The inward-open conformation. **II.** The outward open conformation. **B:** The major conformations in the transport cycle of an MFS-MDR DHA family transporter (N lobe (light blue), C lobe (light orange), substrate shown as red hexagons and proton as green circles) **I.** Inward open loaded **II.** Occluded loaded. **III.** Outward open unload. **IV.** Outward open protonated. **V.** Occluded protonated. **VI.** Inward open deprotonation. **C:** LmrP transport model based on previously obtained result. **I.** Resting state: the resting state is an occluded conformation in which protons cannot enter but allowing the substrate (green hexagon) to enter via the bilayer. **II.** Substrate entrance: binding of the substrate stabilizes the outward-open conformation. **III.** Proton entrance: by opening the extracellular side, the entrance of the ligand triggers the entrance of protons. **IV.** Substrate release: the entrance of protons or water molecules in the binding site leads to the protonation of carboxylic residues. The affinity for the substrate decreases and it is then released. **V.** Conformational change: protons are transferred through the protein via a network of residues. Protonation of a specific residue triggers the conformational switch to the outward-closed/inward-open conformation. **VI.** Proton release: in the inward-open conformation, some residues are exposed to a neutral pH of the cytoplasm. The deprotonation of a specific residue and the release of the proton on the intracellular side precede the return of the transporter back to its resting state.

To further investigate the proposed model, we have focused our research on the structural studies of two specific MFS transporters, namely LmrP and QacA.

1.6 QacA

The quaternairy ammonium compound efflux pump A (QacA) is one of the multidrug efflux pumps from the major facilitator superfamily (MFS) in *Staphylococcus aureus* [31]. The *QacA* determinant carried by the plasmid pSK1 from *S. aureus* was the first bacterial multidrug resistance gene to be described. It is one of the most prevalent plasmid-encoded QAC-resistance mechanism among gram-positive bacteria. *S. aureus* isolates bearing Qac-efflux pump genes were found mostly in nosocomial environment and showed higher resistance to antiseptic treatment [32].

The protein comprises 514 amino acids and is organized in 14 transmembrane α-helices for a total mass of approximately 55kDa. As a member of the DHA2, two additional TM helices are inserted between the classic DHA1 symmetric helical bundles 1-6 and 7-12. Consequently, TM helices 1-6 and 9-14 in QacA exhibit a pseudo 2-fold symmetry that facilitates rocking-switch movements, enabling alternating access on both sides of the membrane.

QacA exhibits a H+:drug stoichiometry of 2 or higher and displays a broad substrate range encompassing nearly 30 organic monovalent and divalent aromatic cations with antibacterial properties. The substrates chosen for investigation include monovalent compounds like ethidium bromide (EtBr), tetraphenylphosphonium (TPP), rhodamine 6G (R6G), and divalent compounds, 4',6-diamidino-2-phenylindole (DAPI), a diamidine and chlorhexidine (CHX), a divalent quaternary ammonium compound (Figure 5) [33].

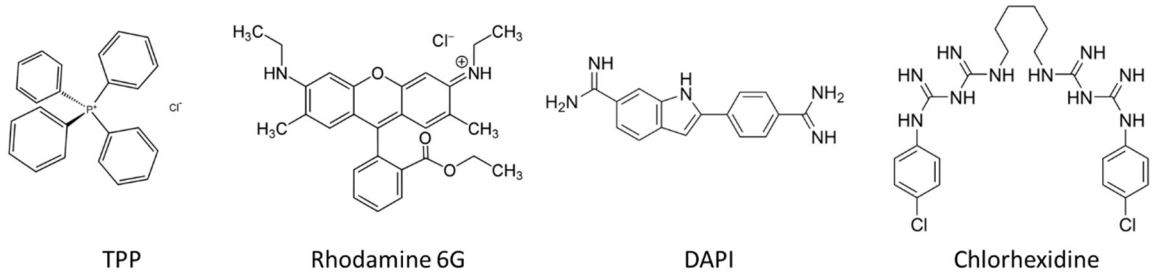

Figure 5: Chemical structures of some substrates of QacA belonging to distinct classes of substances. Are represented here tetraphenylphosphonium (TPP), Rhodamine6G (R6G), 4',6-diamidino-2-phenylindole (DAPI) and chlorhexidine (CHX).

Antiporters typically contain at least one protonatable acidic residue in the TM regions where substrates compete and release protons in the opposite direction to the substrate flow. In the case of QacA, acidic residues have been implicated in triggering conformational changes necessary for substrate binding and release of protons during the transport cycle. Multiple work of mutagenesis (Figure 6A) has led to the conclusion that four residues (D34, D323, D411, and E407) were found to be essential for promiscuous substrate recognition and translocation. These residues are predicted to be located within the transporter, and their mutations resulted in compromised binding and transport properties (Figure 6B).

The acidic residue D34, located in TM 1, was identified as a critical site for substrate recognition. Mutations at this site completely abolished binding and transport abilities. Despite a prediction of a minimal solvent accessibility, D34 could still interact with smaller lipophilic cations, suggesting its importance in substrate recognition [34].

Acidic residues E407 and D411, situated in TM 13, were found to have distinct roles in substrate efflux. While mutations at D411 affected the transport of specific substrates (EtBr and TPP), mutations at E407 showed differential effects. The E407A mutant lost the ability to transport several substrates, whereas the E407Q mutant retained transport activity. This suggests that E407 acts as a protonable site and its mutation decouples substrate efflux from pH gradient dependence [35].

QacB, a paralog of QacA, possesses a natural substitution of D323 to alanine and is unable to transport divalent substrates [36]. This residue was later found to have conditional importance. Mutations at D323

compromised the binding and transport of certain divalent antibacterial compounds, but its role in transporting dequalinium was retained.

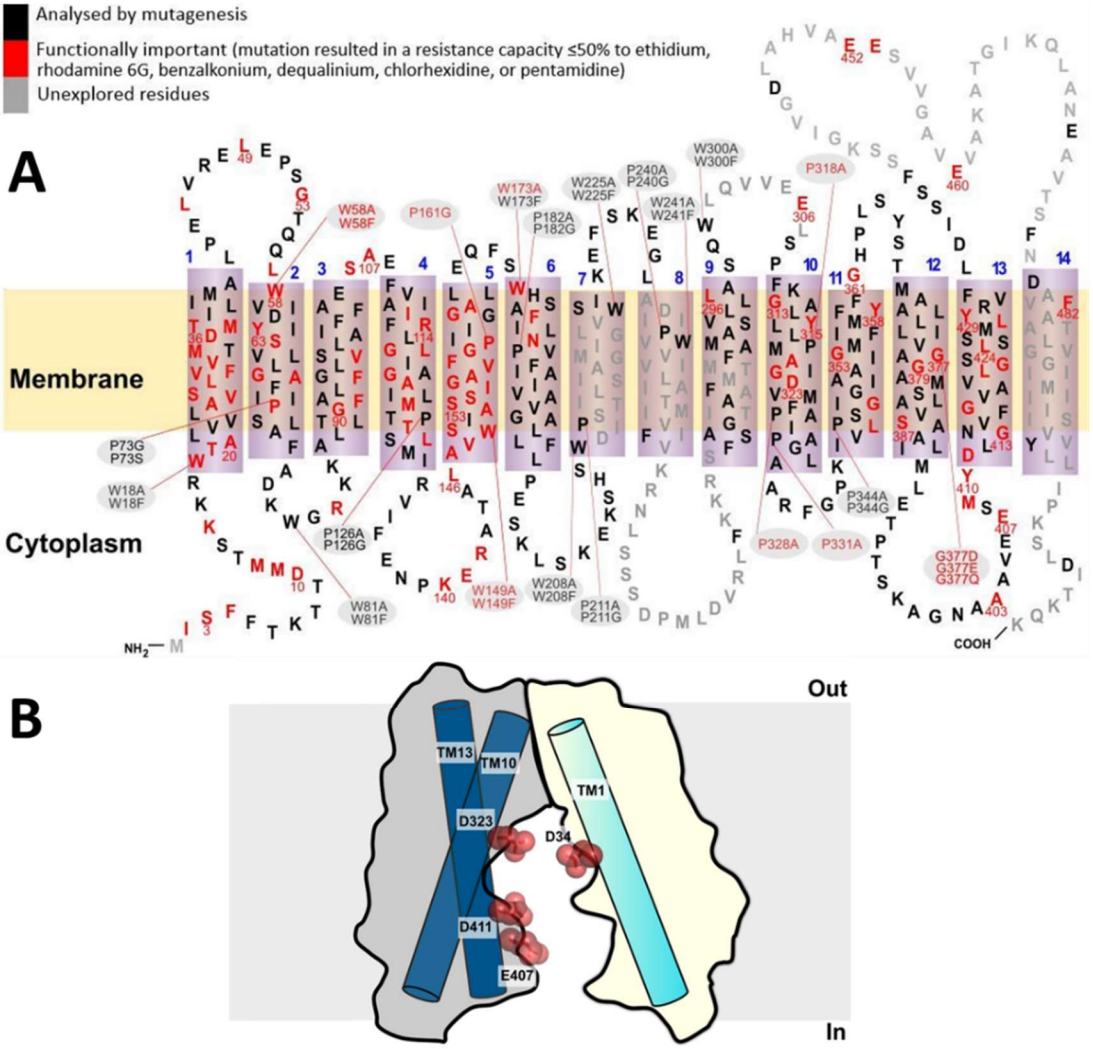

Figure 6: Important residues for QacA's ability to transport. [34] **A:** Primary structure of QacA and representation of the predicted transmembrane helices and loops with functionally important residues colored in red, non-functionally important residues in black and not studied residues in grey. All the tested amino acids were substituted to cysteine or else it is noted on the figure. Functionally important residues were determined by mutation that resulted in a resistance capacity of less than 50% compared to the wild type QacA to either Ethidium, rhodamine 6G, Benzalkonium, dequalinium, chlorhexidine or pentamidine. These MIC values were determined by a standard agar dilution method where *E. coli* DH5a cells expressing wild-type and mutant QacA proteins were grown on LB agar supplemented with different concentrations of individual compounds [Performed by our collaborator at Flinders University]. **B:** Cartoon depicting the acidic residues present in the transport vestibule of the predicted QacA structure that are required for recognition and act as protonation sites helping in transport of the lipophilic cationic ligands [34].

Aromatic amino acid residues play a crucial role in the transport function of multidrug transporters by participating in multidrug binding. In the case of QacA, it has been observed that the aromatic residue Y410 is essential for proper protein function. Y410 was hypothesized as involved in π-stack interactions with aromatic ring present in various substrates, which contribute to substrate binding and transport [35].

A recent article was published on the structure of QacA unraveled by Cryo-EM [133]. As QacA structure obtained in the laboratory was studied prior to the article publication, the article will only be mentioned in the discussion (see Chapter 4.2.3).

1.7 LmrP

Lactococcus lactis multidrug resistant protein (LmrP) is a secondary transporter from the MFS family. Identified in 1995 by genetic studies of the drug extrusion system of a gram-positive bacterium *Lactococcus Lactis*. It is composed of 408 amino acids in 12 TM and has a total molar mass of 45kDa. Sequence of LmrP with secondary structure assigned based on crystal structure is shown below (Figure 7).

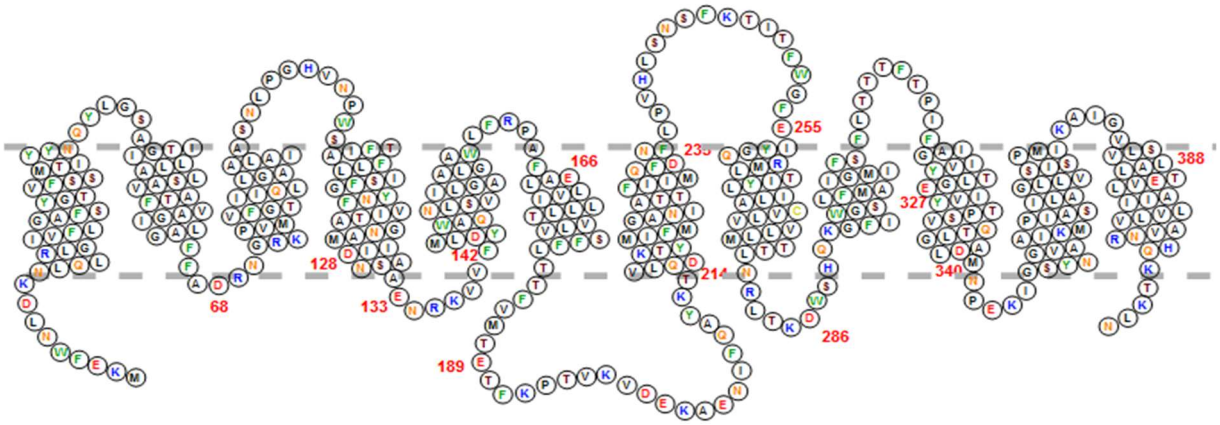

Figure 7: Primary structure of LmrP and representation of the transmembrane helices and loops. Residues colored in red are negatively charged, in blue positively charged and in green aromatic. Both termini are located into the cytoplasm.

The diverse list of substrates transported by LmrP has been studied by monitoring the growth rate of *E. coli* cells in presence of different classes of antibiotics [37]. Besides antibiotics such as lincosamide (clindamycin) and tetracycline, LmrP can also transport DNA intercalating dyes such as Hoechst33342 and EtBr. Even if there is a wide range of substrates (Figure 8), we can identify some similarities between them. The molecules transported are cationic and hydrophobic and their amphiphilic character makes them able to integrate into the lipidic layer [38].

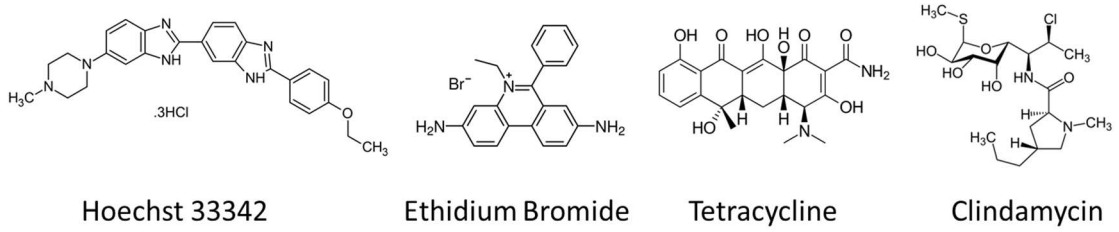

Figure 8: Chemical structures of some substrates of LmrP belonging to distinct classes of substances. Are represented here intercalating agent: Hoechst33342 and EtBr. Antibiotic classes: Tetracycline (tetracycline) and Lincosamides : Clindamycin. Other antibiotic classes are not represented here such as: Lincosamides (Clindamycin), Macrolides (Erythromycin) and Streptogramins (Dalfopristin).

1.7.1 Structure

The structural details of LmrP has been elucidated and published [39] (Figure 9). The crystal structure of LmrP in complex with Hoechst 33342 revealed an outward open (OO) conformation, which was consistent with previous investigations [40]. LmrP exhibited the characteristic canonical fold of the MFS, comprising 12 transmembrane α-helices arranged in two pseudo-symmetrical bundles. The highly conserved 'motif A' [27] between transmembrane helices TM2 and TM3 displayed well-defined features and demonstrated the interaction between Asp68 in TM2 and the bottom of TM11, thereby stabilizing the inward-closed/outward-open state. This motif included residues Arg72 and Asp128, which formed a crucial polar network responsible for the precise positioning of Asp68 towards TM11.

The substrate, Hoechst 33342, exhibits specific polar interactions with negative side chains, particularly Asp235 and Glu327, which had previously been identified as essential for binding [41]. Both Glu327 in TM10 and Asp235 were found to interact directly with either the nitrogen from the piperazine group or from the central benzimidazole group respectively. The negative charges provided by Asp235 and/or Glu327 likely contribute to stabilizing the positive charges of the substrate, facilitating its binding. Notably, the ligand does not engage in hydrophobic interactions with aromatic or aliphatic side chains as observed in other multidrug transporters.

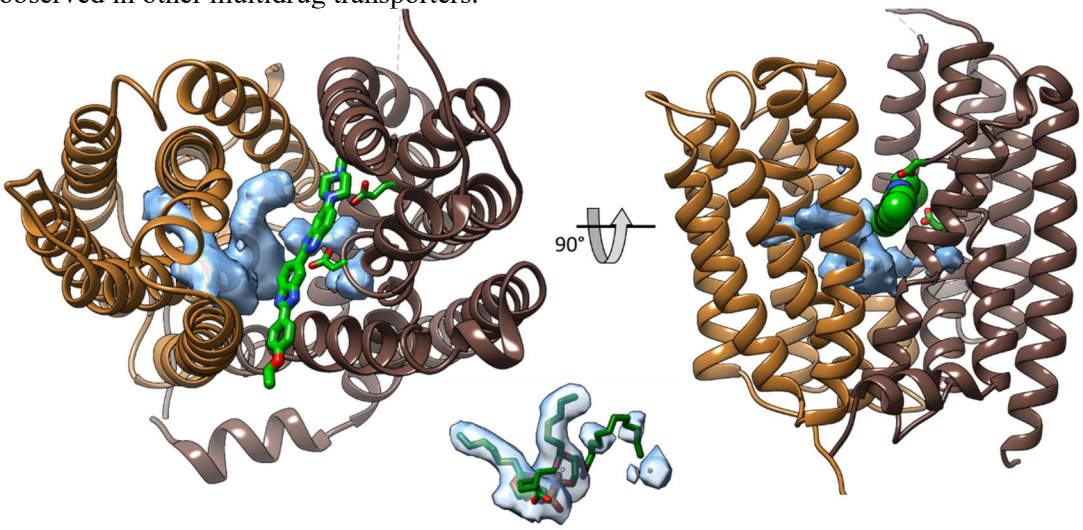

Figure 9: The structure of LmrP in complex with Hoechst 33342 with an observable extra density. Cartoon representation of LmrP, with the N-lobe, central loop and C-lobe colored in brown, dark red and wheat, respectively. Hoechst is represented in green with E327 and D235 pointing towards the substrate. The extra density is visible in the structure and a PG has been modeled inside. [39]

Furthermore, an intriguing finding emerged regarding the presence of a lipid molecule within the substrate-binding pocket of LmrP, situated in close proximity to the bound Hoechst 33342. Molecular dynamics (MD) simulations suggested that a negatively charged lipid, such as phosphatidylglycerol (PG), is anticipated to confer stability to the protein in its substrate-bound conformation. Native mass spectrometry (MS) analysis corroborated the existence of a tightly bound PG molecule, which was observed to be absent upon a point mutation in the binding pocket of the embedded lipid as observed in the X-ray structure. Functional investigations demonstrated that mutations impacting the binding or positioning of this lipid resulted in specific alterations in transport activity depending on the substrate. Disruption of the embedded lipid appeared to diminish the efflux of certain ligands (Hoechst 33342, tetracycline, and to a lesser extent, erythromycin) mediated by LmrP, while the activity towards clindamycin and other ligands remained unaffected.

1.8 Conformational stabilizers

Since only the outward open state of LmrP has been obtained by crystallography, we investigated the possibility of constraining the protein into a specific state with different tools. Doing as such, we could control the conformation state of an MFS to obtain multiple structures and depict a clearer cycle of transport. To study the conformational change of membrane transporters multiple techniques are available. Pulsed electron-electron double resonance spectroscopy (PELDOR/DEER) [42] and single-molecule Förster resonance energy transfer spectroscopy (smFRET) [43] are powerful techniques for investigating conformational changes, structural heterogeneity, and inter-probe distances within biological macromolecules. They provide qualitative information that facilitates mechanistic understanding of biochemical processes and quantitative data for structural modelling.

smFRET is based on the principle of FRET, a non-radiative energy transfer process between two fluorescent molecules, a donor, and an acceptor. In smFRET, the donor and acceptor molecules are attached to different regions of a protein and their distance is measured by monitoring the energy transfer between them. The energy transfer efficiency is sensitive to the space between the donor and acceptor and the degree of energy transfer can be used to deduce the distance between the two molecules. By measuring the distance between the donor and acceptor, smFRET can provide real-time information on the conformational changes of the protein. smFRET can be performed on freely diffusing molecules or on fixed molecules, making it a valuable tool for studying protein dynamics and conformational changes under different conditions [44].

DEER is a technique used to study the motion and structural dynamics of proteins. It is based on the principle of electron spin resonance (ESR) and is used to measure the distance between electron spin-labeled sites on a protein. The target macromolecule is labeled with nitroxide radicals, which are incorporated onto the protein through site-directed spin labeling techniques. Once the spin-labeled protein is prepared, the dipolar coupling between the spins is measured by ESR spectroscopy. The dipolar coupling strength depends on the space between the electron spins and can be used to determine the distance between spin-labeled sites. The technique is sensitive to small changes in distance, typically on the order of 1-2 Å, which allows for the detection of subtle conformational changes in proteins [45].

smFRET and DEER measurement can be used to cross-confirm the conformational landscape of a protein. Each has their own advantage and inconvenience. smFRET measurements are performed on a single particle free in solution compared to the average frozen sample of the DEER method but the distance resolution of the latter is superior. In some rare cases, inconsistencies can occur between the two methods and are mostly attributed to cryoprotectant in the DEER technique and to label protein interaction in smFRET [46]. In our laboratory, both techniques have been used on LmrP and have identified multiple conformational stabilizers that allows us to shift equilibrium between different states of the transport cycle.

In the next chapters, we will present a comprehensive review of the pre-existing findings from our laboratory regarding LmrP, which demonstrate the efficacy of a particular condition in stabilizing a specific conformation. It is important to note that all these results were obtained prior to the commencement of the present study.

1.8.1 Modulation by protonation

It is worth emphasizing that the pH level can exert a substantial influence on the protein. The ionizable groups found in the amino acid residues possess different protonation state depending on the pH. Consequently, pH variations can induce conformational rearrangements and modulate the pKa values of ionizable residues. These changes can have implications for the protein's stability, enzymatic activity, and interactions with other molecules. The pH dependence of a protein can be investigated by evaluating alterations in its tertiary and quaternary structure, as well as by assessing internal motion dynamics within the protein in different acid or basic solutions. [47].

Considering that LmrP operates based on a proton gradient, the influence of pH on the protein's conformational landscape becomes apparent [40]. In the case of *L. lactis*, under physiological conditions, a pH difference of 0.5 is typically observed between the conserved intracellular pH 7 and extracellular usually slightly acidic environment (pH 6.5 or less).

To investigate the impact of pH on LmrP, DEER measurements have been conducted using probes positioned on both the extracellular and intracellular sides of the protein.