CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Biological and Medical Sciences

Searching for a rational strategy in combat with multi-drug resistant staphylococci – options of combination therapy

Diploma thesis

Kamila Kováčová

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Supervisor: RNDr. Klára Konečná, Ph.D.

I hereby declare this thesis as my original work composed independently by myself (under the guidance of my consultant). All sources of literature and images used in this thesis are referenced in citations and properly cited in the text. This thesis has not been used to obtain different or the same degree.

V Hradci Králové   Kamila Kováčová

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ABSTRACT

Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Department of Biological and Medical Sciences

Study program: Pharmacy

Author: Kamila Kováčová

Supervisor: RNDr. Klára Konečná, Ph.D.

Title of the diploma thesis: **Searching for a rational strategy in combat with multi-drug resistant staphylococci – options of combination therapy**

Background

With the rise of multi-drug resistance in microbes, treating many infectious diseases has become challenging. Among clinically important bacteria with higher priority, pathogens from the ESKAPE group are included. The S in the acronym ESKAPE stands for *Staphylococcus aureus* (*S. aureus*). This bacterial agent is a common human pathogen and can cause various infectious diseases. *S. aureus* strains are often resistant to antibiotics such as β-lactams, chloramphenicol, lincomycin, aminoglycosides, tetracyclines, macrolides, sulfonamides, and rifampicin. The need for new antibiotics or alternative strategies to combat infections caused by multi-drug-resistant pathogens has become more apparent in recent years. A combination therapy could cover the requirement for an alternative treatment strategy. Although it is already used in clinical practice, it is mainly due to empirical knowledge, and proven evidence about treatment benefits or potential pitfalls is lacking.

Aim

This diploma thesis is focused on evaluating the mutual interaction and impact on the activity of selected commercially available antibiotics in combinations. The *Staphylococcus aureus,* MRSA, American Type Culture Collection (ATCC) 43300, CCM 4750, purchased from the Czech Collection of Microorganisms (CCM) was used to determine the effect of selected pair-wise combinations. Selected antibiotics for this thesis were ciprofloxacin (CIP), cotrimoxazole (COT), daptomycin (DAP), linezolid (LIN), rifampicin (RIF), tigecycline (TIG), and vancomycin (VAN). Combinations that show promising results will be recommended for further testing.

Methods

A universal bipolar solvent dimethylsulfoxide was used to prepare a stock solution of selected antibiotics. Cation-adjusted Müller-Hinton broth was used as a medium for a final antibiotic solution and bacterial suspension.

The checkerboard microdilution method was applied to assess the interaction of antibiotics in combinations. Spectrophotometric measurement was used to determine the degree of inhibition of bacterial growth. The potency of the antibiotic pair-wise combinations was expressed by creating a heat map of each combination using a percentage of inhibition values, and the categorization of mutual antibiotic drug interactions was determined by calculating the FIC (fractional inhibitory concentration) index.

Results

Seventeen pair-wise combinations, each comprising of thirty-six sub-combinations, were evaluated. The result of the evaluation of most pair-wise antibiotic combinations was indifference. One combination expressed an outright antagonistic effect, and two others expressed indifference bordering on antagonism. Two combinations, which showed mostly indifference, had a small number of sub-combinations where the additive effect was registered. Two combinations expressed additive effect.

Conclusion

In summary, out of seventeen evaluated pair-wise antibiotic drug combinations, two combinations, namely CIP+RIF and COT+RIF, expressed promising mutual interaction (additive effect) in at least three drug concentration ratios. These drug combinations will undergo further advanced assessments— they will be incorporated into antimicrobial cocktails (e.g., with antimicrobial peptides, efflux pump inhibitors, or biosurfactants), and the antibiofilm activity will also be studied.

Keywords: MRSA, combination therapy, *in vitro* susceptibility testing, checkerboard microdilution method, drug combinations interactions

ABSTRAKT

Univerita Karlova v Prahe

Farmaceutická fakulta v Hradci Králové

Katedra biologických a lékařských věd

Študijný program: Farmácia

Autor: Kamila Kováčová

Školiteľ: RNDr. Klára Konečná, Ph.D.

Názov diplomovej práce v slovenčine: **Zavedenie racionálnej stratégie na boj proti multirezistentným stafylokokom - možnosti kombinačnej terapie**

Názov diplomovej práce v češtine: **Nastolení racionální strategie v boji s multirezistentními stafylokoky - možnosti kombinační terapie**

Úvod

Liečba mnohých infekčných ochorení sa stala náročnou spolu so vzrastom výskytu multirezistentných mikróbov. Medzi klinicky relevantné mikróby patria aj patogény zo skupiny ESKAPE. Písmeno S v akronyme ESKAPE označuje *Staphylococcus aureus* (*S. aureus*). Tento mikrób je bežným ľudským patogénom, a môže spôsobiť niekoľko rôznych infekčných ochorení. Kmene *S. aureus* sú často rezistentné na antibiotiká ako sú β-laktámy, chloramfenikol, linkomycín, aminoglykosidy, tetracyklíny, makrolidy, sulfonamidy, a rifampicín. Núdza o nové antibiotiká nebo alternativne stratégie na boj proti infekciám spôsobeným multirezistentnými mikróbmi sa za posledných pár rokov stala očividnejšou. Požiadavky na alternatívnu statégiu môžu byť pokryté využitím kombinačnej terapie. Napriek tomu, že sa táto stratégia už v klinickej praxi používa, je to skôr kvôli empirickým znalostiam, a preukázateľné dôkazy o výhodách alebo možných úskaliach takejto terapie sú nedostatočné.

Cieľ

Táto diplomová práca je zameraná na hodnotenie vzájomného účinku a vplyvu kombinácií vybraných komerčne dostupných antibiotík na ich antibakteriálnu aktivitu. Kmeň *Staphylococcus aureus,* MRSA, American Type Culture Collection (ATCC) 43300, CCM 4750, kúpený z České sbírky mikroorganizmů (CCM), bol použitý na zistenie účinnosti predom zvolených dvojkombinácií antibiotík. Vybrané boli antibiotiká ciprofloxacín (CIP), kotrimoxazol (COT), daptomycín (DAP), linezolid (LIN), rifampicín (RIF), tigecyklín (TIG), a vankomycín (VAN). Kombinácie, ktoré vykazújú sľubné výsledky, budú odporúčané na ďalšie testovanie.

Metodika

Na prípravu zásobných roztokov jednotlivých antibiotík bolo použité univerzálne bipolárne rozpoúšťadlo dimetylsulfoxid. Katiónovo upravený Müller-Hintonov bujón bol použitý ako

médium pre finálny roztok antibiotík a pre bakeriálnu suspenziu. Na posúdenie účinku kombinacií antibiotík bola použitá checkerboard mikrotitračná metóda. Prítomnosť bateriálneho nárastu bola určená pomocou spektrofotometrického merania. Účinok dvojkombináce antibiotík sa určil pomocou vytvorenia heat mapy každej kombinácie, a výpočtom FIC (frakcionálna inhibičná koncentrácia) indexu sa určil character interakce antibiotík.

Výsledky

Bolo hodnotených sedemnásť dvojkombinácií, z toho každá pozostávala z tridsaťšesť možných podkombinácií. Väčšina kombinácií bola vyhodnotená ako indiferentné. Jedna kombinácia vykazovala priamo antagonistický efekt, a dve ďalšie kombinácie vykazovali indiferenciu hraničiacu s antagonizmom. Dve kombinácie, ktoré vykazovali väčšinou indiferentný efekt, mali malé čislo podkombinácií, ktoré vykazovali aditívny efekt. Dve kombinácie preukázali aditívny efekt.

Záver

V súhrne, dve zo sedemnástich hodnotených dvojkombinácií antibiotík, konkrétne CIP+RIF a COT+RIF, vykazovali sľubnú vzájomnú interakciu (aditívny efekt) v aspoň troch koncentračných pomeroch. Kombinácie týchto liekov podstúpia ďalšie pokročilé hodnotenia—budú začlenené do antimikrobiálnych koktejlov (napr. v kombinácii s antimikrobiálnymi peptidami, inhibítormi efluxných púmp, alebo biosurfaktantmi), a taktiež bude skúmaná ich aktivita proti bakteriálnemu biofilmu.

Kľúčové slová: MRSA, kombinačná terapia, testovanie citlivosti *in vitro*, checkerboard mikrotitračná metóda, interakcie kombinácií liekov

TABLE OF CONTENT

1. INTRODUCTION

The stagnant development of new antibiotics, together with the spread of multidrug-resistant (MDR) bacteria, has brought on a health crisis– a lack of effective antimicrobials. The World Health Organization (WHO), together with the U.S. Centers for Disease Control and Prevention (CDC), categorizes MDR as an imminent threat to human health. Although the occurrence of MDR genes happens naturally in the environment, due to the lack of rapid diagnostic methods, and unnecessary use of broad-spectrum antibiotics creating selective pressure, the possibility of their survival only increases. (1)

In response to the rising emergence of MDR pathogens, the WHO has listed several bacteria that show resistance to conventional treatment and are considered dangerous. Among these pathogens, high priority was given to methicillin-resistant *Staphylococcus aureus* (MRSA). (2)

MRSA is considered a „superbug", and causes several types of infections, going from mild skin infections to severe infections such as osteomyelitis, lung abscesses, meningitis, or pneumonia. Among the most dangerous infections is also infective endocarditis, having the highest mortality and morbidity in comparison to other infections caused by MRSA. (3)

Considering the severity of infections caused by MRSA and limited treatment options, it is apparent that new antimicrobials or new, effective alternative antimicrobial strategies (such as photosensitizers) are needed. However, the process of introducing new compounds is long and strenuous. (4)

Combination therapy is one possible strategy to combat MDR pathogens. The combined effectivity of antibiotics might be beneficial for difficult-to-treat, deep-seated, or persistent bacterial infections. However, the antimicrobial agents cannot be combined haphazardly, and instances such as augmented adverse effects, increased toxicity, or mutual interaction of selected antibiotics need to be taken into consideration. (5)

To accurately select a promising combination of antimicrobial drugs to combat infections caused by MRSA, the *in vitro* testing method was employed in this thesis, and seven commercially available antibiotics and their respective pair-wise combinations were evaluated.

2. AIM

Combination antimicrobial therapy represents one of the alternative strategies for combatting difficult-to-treat infections caused by MRSA. Antimicrobial drugs with different targets in the microbial cell are preferred, but not necessary. The strategy to hit multiple targets in one action contributes to reducing the risk of the emergence and spread of MRSA. Revealed enhanced (synergistic) activity in selected drug-drug combination(s) allows for lowering the dose of a single drug and reducing the risk of dose-related antimicrobial toxicity. These drug combinations could create the basis for rational combination therapy of complicated staphylococcal infections, especially those associated with staphylococcal biofilm formation. Nevertheless, this hypothesis needs to be proven by *in vivo* (cyto)toxicity testing.

This diploma thesis is focused on the evaluation of the antibacterial effect of selected pair-wise combinations of antibiotics. In total, 17 pair-wise combinations of pre-selected antibiotics were tested to determine whether their mutual interaction leads to a synergic effect. For the *in vitro* screening of mutual interactions of two drugs in combination, the laboratory reference strain, MRSA ATCC 43300 was employed. The checkerboard assays with microdilution arrangement, and spectrophotometric measurement were employed in this work.

Based on the results, promising combinations will be recommended and employed for drug cocktail composition (combination with inhibitors of efflux pumps/antimicrobial peptides). Pair-wise drug combinations and cocktails will be subjected to further testing, such as evaluation of the impact of drug cocktail on toxicity *in vivo* (employment of alternative model *Galleria mellonella*), evaluation of PAE (post-antibiotic effect) and PAE-SME (postantibiotic sub-MIC effect), and study of tolerance/resistance development towards antibiotic treatment (sub-inhibitory concentration treatment and regrowth experiments).

3. THEORETICAL PART

3.1. The issue of multidrug-resistant pathogens

The use of antimicrobials as weapons against pathogens is a standard practice. Antimicrobial compounds in their therapeutic concentrations express either bactericidal or bacteriostatic potential in the treatment of infections. However, exposing pathogens to sub-clinical concentrations of antimicrobials can cause a selective pressure and survival of pathogens with genetic advantages, or can result in more genome changes. All these factors contribute to the spread of resistant bacteria. (6)

The occurrence of multidrug-resistant (MDR) genes happens naturally in the environment, as well as due to the acquisition of resistance genes (i.e. by horizontal gene transfer such as transduction, transformation, or conjugation) (7). Due to the lack of rapid diagnostic methods and unnecessary use of broad-spectrum antibiotics, the selection of pathogens possessing MDR genes caused their increase in microbial population (1). Anytime new resistance has come to light, solutions like modification of existing antibiotics with limited cross-resistance, or introduction of newer antibiotics have been used (6). This approach could have been effective, were it not for the overuse of antimicrobials in health care and agriculture, leading to their unnecessary circulation in the environment, and combined with inappropriate handling (such as frivolous prescription of broad-spectrum antibiotics, inappropriate dosing, poor treatment adherence, prophylactic use of antimicrobials in agriculture and husbandry), the antimicrobial resistance continues to rise. (8)

In recent years, the definition of multidrug resistance (MDR) has changed. These changes have been reflected in most conducted studies and were adopted from the classification proposed by the US and European Center for Disease Control and Prevention (CDC and ECDC). Multidrug-resistant (MDR, pathogen resistant to one or more antibiotic agents from three or more classes), extensively drug-resistant (XDR, pathogen susceptible to one or two antibiotic classes only), and pan-drug-resistant (PDR, pathogen resistant to antibiotic agents from all classes) pathogen are employed now. (9; 10; 11)

Although no systematic international surveillance of MDR has been conducted, it is estimated that, per year, hospital-acquired (HA) and community-acquired (CA) MDR infections are responsible for over 33 000 deaths and 874 000 disability-adjusted life years (DALYs) in Europe alone, which accounts for $1.5 billion in expenditures (1). Despite the growing threat of such „superbugs" widespread, the perfunctory effort to prescribe correct antimicrobials is rather irritating (9).

3.2. Pathogens from the group ESKAPE

Considering the worrying reality we are facing, the WHO issued a list of priority-status pathogens. The name ESKAPE is an acronym for a group of both gram-negative and gram-positive bacteria, namely: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. (1; 2; 12)

They are the most common cause of life-threatening nosocomial infections, especially for critically ill or immunocompromised patients. Due to their drug resistance mechanisms, these pathogens can „escape" the bactericidal/bacteriostatic effect of antimicrobial drugs, rendering conventional therapies ineffective. This accounts for increased morbidity and/or mortality of patients, as well as expenses in health care. (9; 13)

Figure 1*: List of ESKAPE pathogens, together with a pictorial representation of places with their natural occurrence in the human body and infection-affected areas. Source: Pulgar, et al.; The ESKAPE bacteria group and its clinical importance; 2019.* (13)

3.2.1. Resistance of ESKAPE pathogens

ESKAPE pathogens display resistance against oxazolidinones, lipopeptides, macrolides, fluoroquinolones, tetracyclines, β-lactams (with or without β-lactamase inhibitors), and last-line antibiotics like carbapenems, glycopeptides, polymyxins (14). Typical mechanisms of resistance are depicted in *Figure 2*.
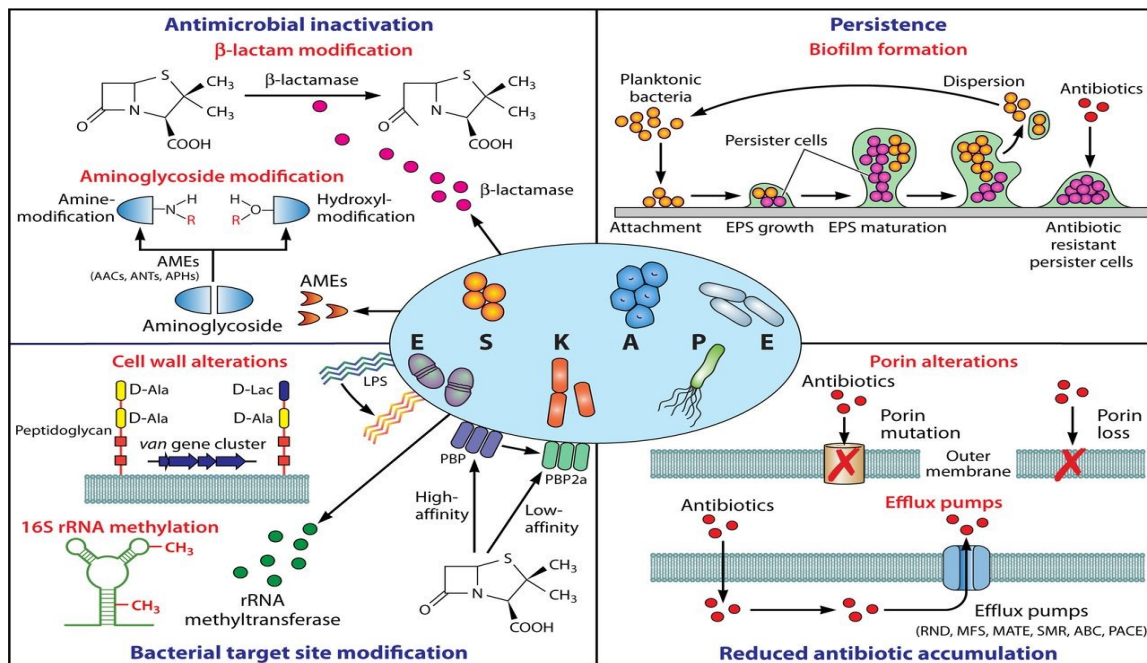
Figure 2: *Schematic representation of mechanisms of resistance in ESKAPE pathogens. Mechanisms of resistance are divided into four categories: 1.) antimicrobial inactivation mediated by enzymes: irreversible destruction of the active antibiotic site (e.g. hydrolytic cleavage of the β-lactam ring by β-lactamases), covalent modification of principal scaffold structures of the antibiotic drugs, hindering interaction between drug and bacterial target site (such as aminoglycosides modifying enzymes that catalyze the modification at hydroxyl- or amino groups of drug molecule); 2.) formation of biofilm, which hinders access of antimicrobial compound to bacterial cells; 3.) modification of antibiotic target sites, reducing the drug's binding affinity to surface cell targets (e.g. expression of PBP2a gene leading to reduced affinity of β-lactam antibiotics) or to intracellular cell targets, e.g. methylation of 16S RNA subunit; 4.) reduced accumulation of antibiotic drug caused by mutation of porins in outer membrane or by their loss (e.g. in bacteria P. aeruginosa, A. baumanii), and overexpression of efflux pumps to extrude antibiotic drugs out of bacteria (e.g. families of efflux pumps RND, MFS, MATE, SMR, ABC, and PACE); Legend: AACs= aminoglycoside acetyltransferases; ABC= ATP-binding cassette; AMEs= aminoglycoside-modifying enzymes; ANTs= aminoglycoside nucleotidyltransferases; APHs= aminoglycoside phosphotransferases; EPS= extracellular polymeric substance; LPS= lipopolysaccharide; MATE= multidrug and toxic compound extrusion; MFS= major facilitator superfamily; PACE= proteobacterial antimicrobial compound efflux; PBP= penicillin-binding protein; RND= resistance-nodulation-division; SMR= small multidrug resistance. Source: De Oliveira, et al.; Antimicrobial Resistance in ESKAPE Pathogens; 2020* (1)

3.2.2. Strategies to combat infections caused by ESKAPE pathogens

Even though the research of new antibiotics against ESKAPE pathogens has been constantly conducted, since the beginning of the 1990s, a limited number of innovations have been introduced. Out of 11 new antimicrobials introduced between 2017 and 2019, only five have been approved by the European Medicines Agency (EMA), namely: delafloxacin (Baxdela/Quofenix), eravacycline (Xerava), the imipenem-cilastatin-relebactam combination (Recarbrio), the meropenem-vaborbactam combination (Vaborem), and ceftobiprole (Zeftera). (1)

The projects aimed at the research of new combat strategies against ESKAPE pathogens can be divided into several groups: direct-acting agents and potentiators of direct-acting drugs, antibodies and vaccines, phages and phage-related products, microbiota-modulating therapies, antivirulence approaches, repurposed drugs, immunomodulators, and others. (15).
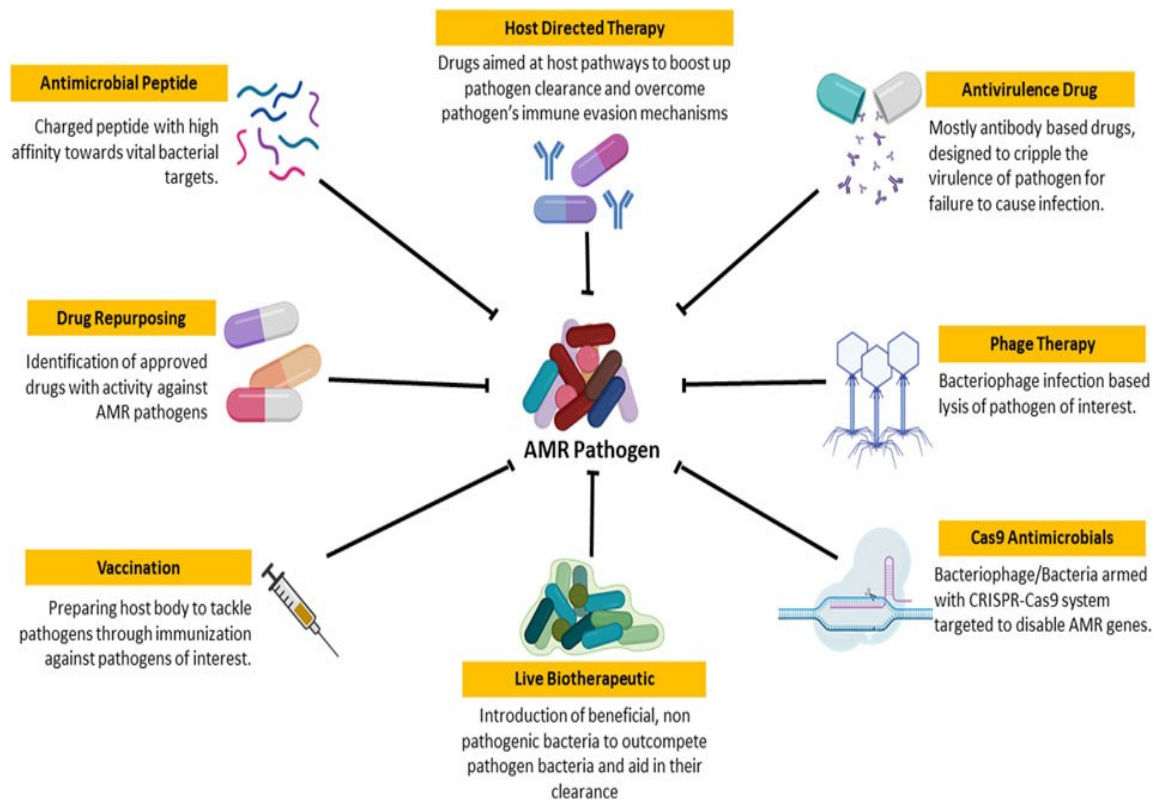
Figure 3: *Schematic representation of alternative strategies to combat AMR pathogens. Legend: AMR= antimicrobial-resistant, CRISPR Cas9= Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9. Source: Bhandari, et al.; Next-Generation Approaches Needed to Tackle Antimicrobial Resistance for the Development of Novel Therapies Against the Deadly Pathogens; 2022* (16)

3.3. *Staphylococcus aureus*

3.3.1. Etiology, epidemiology, and pathogenesis of infection

Staphylococcus aureus (S. aureus) is a non-motile, spherical bacterium from the family *Staphyloccocaeae,* which forms clusters in the shape of grapes. It is a Gram-positive, aerobic, and facultative anaerobic pathogen with high clinical relevance. (17; 18; 19; 20; 21)

Staphylococci grown on a blood agar culture display thick, glossy, and round colonies, yellow in color, with a size around 1-2 mm in diameter. These bacteria belong to coagulase-positive, catalase-positive, and oxidation-negative microorganisms. Most strains of *S. aureus* demonstrate a hemolytic activity. (22; 23)
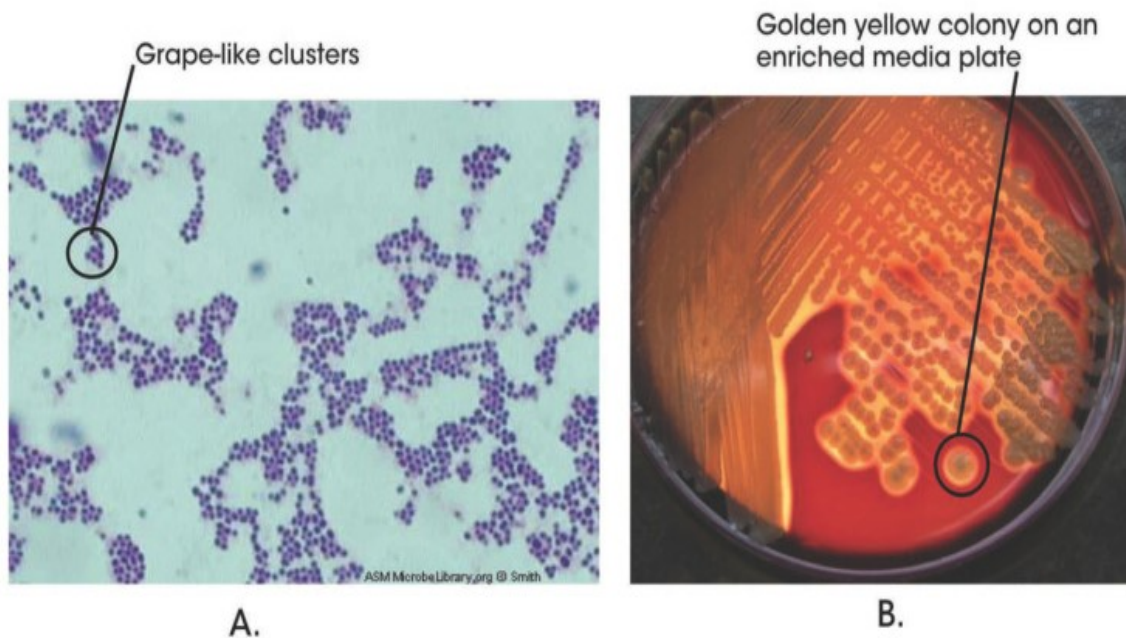
Figure 4: *Depiction of Staphylococcus aureus, A= microscopic representation, B= phenotypic representation on blood agar. Source:* FAQ: The Threat of MRSA - NCBI Bookshelf (nih.gov)

S. aureus is commonly found in the human microbiome, specifically on the skin surface or nasal mucosa. However, due to the opportunistic nature of *S. aureus*, when even a small fissure or any kind of disruption occurs (e.g. surgical wounds, chronic skin lesions), *S. aureus* gains access to soft tissue and bloodstream and can cause infection. (17) Risk factors linked to MRSA infection are long-term hospitalization, patients admitted to an intensive care unit or nursing home, recent administration of antibiotics, surgical or invasive procedures (insertion of a catheter, surgical wounds), hemodialysis, and immunodeficiency (e.g. HIV infection) (3).

S. aureus can produce toxins like enterotoxins, toxic shock syndrome toxins, or exfoliantin. Strains producing these toxins can be a causative agent for staphylococcal foodborne disease, scalded skin syndrome, or toxic shock syndrome. Further, *S. aureus* can cause non-life-threatening, but debilitating conditions and minor skin infections (such as impetigo, pimples, boils, abscesses, etc.), but also life-threatening, systematic infections like meningitis, pneumonia, endocarditis, bacteremia, and brain sepsis. (18; 24; 25)

Since *S. aureus* infections leading to bacteremia are often transmitted in healthcare settings, preventive measures (like thorough sanitization or antimicrobial prophylactic treatment of patients undergoing high-risk procedures), have been implemented. The economic burden of treating *S. aureus* bacteremia is also very high, considering the recurring or prolonged hospitalizations, the cost of needed surgical procedures in case of infections from the implanted prosthesis and possible loss of implant, rehabilitation, and lastly, the decrease of patient's quality of life and prolonged sick leave. (26; 27) Infections caused by *S. aureus* are problematic because of antibiotic resistance among its isolates, among which infections caused by MRSA are considered to be the most significant in clinical practice. MRSA infections are characterized by higher mortality, morbidity, and prolonged hospital stay, in comparison to infections caused by methicillin-susceptible *S. aureus* (MSSA). (18)

3.3.2. Methicillin-resistant *S. aureus* (MRSA)

S. aureus displayed its ability to develop resistance to antibiotics soon after their introduction. Just two years after the introduction of penicillin in the 1940s, *S. aureus* resistance to penicillin was detected. In 1959, methicillin was introduced as one of the first semi-synthetic β-lactam antibiotics against staphylococcal infections. However, *S. aureus* managed to build up resistance against methicillin within one year of its introduction into clinical practice. (28; 29)

After the introduction of methicillin, the prescription of this drug was initially widespread. However, methicillin has rather high toxicity, and ever since more stable β-lactam antibiotics became available (such as oxacillin, flucloxacillin, and dicloxacillin), methicillin became an obsolete antibiotic. Even though methicillin is no longer used, the term MRSA persisted and now refers to a type of *S. aureus* strain that is resistant to β-lactam antibiotics like penicillin, amoxicillin, oxacillin, and methicillin. (17; 20) The European Committee on Antimicrobial Susceptibility Testing (EUCAST) defines MRSA as an *S. aureus* strain with oxacillin MIC breakpoint value > 2 mg/L (30). Staphylococcal cassette chromosome *mec* (SCC*mec*) is a mobile genetic element that carries methicillin-resistant genetic component A (*mec*A), which gives the pathogen the capacity to produce a penicillin-binding protein 2A (PBP2a), which reduces affinity for binding for almost every β-lactam antibiotic. However, besides *mec* genes, other chromosomally determined factors are essential for the expression of methicillin resistance— *fem* genes are recognized as additional chromosomally determined factors, playing an important role in methicillin resistance in *S. aureus.* (25; 28; 31) By acquisition of SCC*mec* components, MSSA can turn into MRSA (20).

In addition to methicillin (and other penicillin-like antibiotics) resistance, MRSA can also acquire resistance to the combination of β-lactams with inhibitors of β-lactamase (clavulanic acid, sulbactam), and several other classes of antibiotics, such as cephalosporins, macrolides, tetracyclines, aminoglycosides, fluoroquinolones. In most cases, multidrug-resistant MRSA (MDR-MRSA) is not affected by first-line antibiotics, and multiple studies have stated the resistance of MDR-MRSA to vancomycin, linezolid, and daptomycin. (17; 19)

MRSA quickly became one of the pathogens with frequent occurrence in almost all parts of the world, owing to its rapid spread (22). Individuals with positive MRSA colonization test (i.e. carriers; MRSA is present in their microbiome, but does not evoke a response from the immune system, does not cause damage to cells, nor does it lead to the manifestation of clinical signs and symptoms of infection) have an increased probability of infection occurrence and are also a notable source for interpersonal infection transmission (17).

3.3.2.1. Types of MRSA

There are three types of MRSA strains, which are distinguished by the conditions of their spread and by their molecular type of SCC*mec* components:

- hospital-acquired MRSA (HA-MRSA): these strains contain SCC*mec* types I, II, III, VI, and VIII. (20) SCC*mec* type II and III encode additional resistance determinants, enabling resistance to antibiotics other than β-lactams (21). Thus, most HA-MRSA strains express resistance to other antimicrobials such as aminoglycosides, fluoroquinolones, lincosamides, and macrolides (32). Hospitals are the most common ground for contracting this type of MRSA infection (infected patients, carriers, or contaminated objects (33)), other places where this type of MRSA occurs are facilities for long-term care or facilities offering one-day surgery (34).

- community-acquired MRSA (CA-MRSA): these strains contain SCC*mec* types IV, V, and VII (20). While CA-MRSA strains generally exhibit resistance to β-lactams, they are susceptible to other antibiotics like clindamycin, tetracycline, and trimethoprim-sulfamethoxazole. In CA-MRSA is a high prevalence of the Panton-Valentine leukocidin gene (gene encoding virulence factor, powerful cytotoxin). (21; 34) These strains do not normally occur in healthcare facilities or healthy people without a history of recent hospitalizations or surgical procedures (32; 33). Even though infections caused by CA-MRSA are usually mild in character, severe, deadly infections like pneumonia or sepsis can also occur (21; 34).

- livestock-associated MRSA (LA-MRSA): these strains contain SCC*mec* types IX, X, and XI (20), and can be found mainly among livestock animals (like pigs and horses) mostly due to improper antibiotic use (19; 31; 32). These animals can be a source of MRSA infection, and strains are transmitted to humans who come in close contact with animals (especially professional caregivers and veterinarians) (34).

3.4. Antibiotic therapy for *S. aureus* infections

Antibiotics are the baseline for the treatment of *S. aureus* infections. In the sections listed below, the most important classes of antibiotics and some included drugs used in the therapy of S. *aureus* infections are briefly summarized (including their mode of action, mechanism of resistance, and indication).

3.4.1. Penicillin-like antibiotics and cephalosporins

- **Mode of action:** Penicillin-like antibiotics and cephalosporins (as well as most other β-lactam antibiotics) competitively inhibit penicillin-binding protein (PBP) such as the enzyme *D, D*-transpeptidases and *D, D*-carboxypeptidases, which are responsible for the catalysis of cross-linking of peptidoglycan. This inhibition leads to cell death (35).

- **Mechanism of resistance:** Resistance to penicillin-like antibiotics can be acquired by the following mechanisms:

  o Synthesis of β-lactamases: the resistance results from the inactivation of antibiotics by microbial β-lactamases encoded on the *bla*Z gene, which can be found in several plasmids and transposons. 4 staphylococcal β-lactamases (A-D) are known.

  o Synthesis of PBP2 (methicillin-resistance, see Chapter 3.3.2): resistance to all β-lactam antibiotics, except for ceftobiprole and ceftaroline. (36)

- **Indication:** Penicillinase-resistant penicillins (cloxacillin, oxacillin, flucloxacillin), and cefazolin (first-generation cephalosporin) are usually used as the drugs of choice for infections caused by MSSA. (26; 37)

  o **Ceftaroline** (prodrug ceftaroline fosamil/medocaril): 5th generation of cephalosporin. Effective for infections caused by vancomycin-resistant *S. aureus* (VRSA) and daptomycin-nonsusceptible *S. aureus* (DNSA). It can be used against more serious infections like complicated skin and soft tissue infections (SSTI), endocarditis, community-acquired pneumonia (CAP), and infections associated with the insertion of prosthetics (17; 28; 38; 39; 40), and as salvage therapy for *S. aureus* bacteremia (SAB). (41) Ceftaroline is a favorable option for outpatient parental antimicrobial therapy (OPAT), due to its lower nephrotoxicity and hepatotoxicity, reduced phlebitis rate, and better storage condition. (26)

  o **Ceftobiprole:** 5th generation of cephalosporin, effective against DNSA and linezolid-nonsusceptible MRSA, alternative for the treatment of SAB, CAP, hospital-associated pneumonia (HAP), and SSTI. (17; 26; 28; 38)

3.4.2. Glycopeptides (vancomycin, teicoplanin)

- **Mode of action:** Vancomycin and teicoplanin inhibit the last stage of synthesis of the cell wall. The antibiotic compound binds to the terminal part of the peptidoglycan chain D-ala-D-ala, preventing subsequent transglycosylation and transpeptidation, thereby hindering the integrity of the cell wall. (2; 21; 22; 23; 25; 42)

- **Mechanism of resistance (vancomycin):** Vancomycin-susceptible *S. aureus* (VSSA) is defined as a strain with MIC< 2 mg/L (24). Different types of vancomycin resistance in *S. aureus* were described:

   o vancomycin-intermediate *Staphylococcus aureus* (VISA) is defined as an *S. aureus* strain with vancomycin MIC= 4—8—16 mg/L (42). The overuse of vancomycin, due to the increase in MRSA infections, resulted in decreased susceptibility of *S. aureus* to vancomycin (23; 28). Resistance occurring in VISA strains is not because of the acquisition of resistance genes but due to accumulated chromosomal mutations in determinants of cell wall synthesis altering the cell wall structure, and mutations in the ribosomal gene *rpo*B. This causes the thickening of the bacterial cell wall and the decrease of negative cell surface charge. (2; 11; 23; 42) Infections caused by VISA are often linked with complications like extended hospitalization, persistent infection, and prolonged administration of vancomycin, leading to increased risk of nephrotoxicity, and potential therapy failure. (24)

   o hetero-VISA (hVISA) is a phenotype of VISA characterized as a seemingly homogenous population of *S. aureus* cells, where the majority of cells are susceptible to vancomycin (with MIC< 2 mg/L), and a small subpopulation acts as VISA (with MIC> 4 mg/L and thickened cell wall) (23).

   o slow-VISA (sVISA) is a recently defined type of *S. aureus* strain. The main characteristic is the slow growth of this phenotype— it takes 72 or more hours to establish a colony. MIC for vancomycin in sVISA corresponds to > 8 mg/L, and the sVISA profile of resistance and macromorphology of colonies are unstable— it can revert to VSSA in the event of vancomycin absence. (24; 42)

   o vancomycin-resistant *Staphylococcus aureus* (VRSA) is defined as a *S. aureus* strain with vancomycin MIC≥ 16 mg/L (42). By acquisition of the *van*A-F, *van*G, *van*I, *van*G, and *van*M-N gene clusters the pathogen gains the ability to hydrolyze the precursor, D-ala-D-ala sequence— a terminal part of the peptidoglycan chain. This results in the synthesis of D-ala-D-lac (encoded by *van*A, *van*B, *van*D, *van*F, *van*I, and *van*N— the "VanA"-type resistance, with a higher level of resistance), or D-ala-D-ser (encoded by *van*C, *van*E, *van*G, *van*L, and *van*N— the "VanC"-type resistance, with a lower level of resistance) precursors, to which the molecule of vancomycin is unable to bind. (20; 43) VRSA does not progress from VISA. Transfer of *van*A gene originates from vancomycin-resistant *Enterococcus faecalis* (2). The prevalence of VRSA is low, possibly because of limited space in *S. aureus* cells for

enterococcal plasmid with *van*A gene, or due to incompatibility with methicillin resistance (the so-called "seesaw effect") (42).

- **Indication:**

o Vancomycin is considered a first-line antibiotic for the treatment of serious, and invasive MRSA infections (caused by both CA-MRSA and HA-MRSA), such as pneumonia, bacterial sepsis, infective endocarditis, bacteremia, osteoarticular infections, and severe SSTI (22; 28; 44). However, it appears that vancomycin is less effective for MSSA infections (26; 29).

o Teicoplanin is a structurally similar compound to vancomycin. It can be used as a replacement antibiotic for patients with penicillin or vancomycin intolerance. Clinical use is for SAB, endocarditis, SSTI, and lower respiratory tract infection. (22; 25; 41; 38) The advantages of teicoplanin compared to vancomycin are lower nephrotoxicity and lower vascular toxicity (26; 45).

3.4.3. Lipopeptides (daptomycin)

- **Mode of action:** Daptomycin is a bactericidal, concentration-dependent antibiotic. It is a cyclic lipopeptide with a fatty acid side chain, analogous to cationic antimicrobial peptides (created by the intrinsic immune system) in structure and function (46). Daptomycin is incorporated into the cell membrane of bacteria in the form of a daptomycin-calcium complex, which is easily accepted by the cytoplasmatic membrane (calcium-dependent manner of action). The molecule of daptomycin oligomerizes and creates pores in the cytoplasmatic membrane, which leads to the loss of intracellular ions, depolarization of the membrane, and delocalization of enzymes responsible for cell wall synthesis. This results in destroyed cell wall integrity, ultimately leading to the death of the cell. (2; 20; 22; 29; 38; 42; 45; 47)

- **Mechanism of resistance:** The daptomycin resistance is associated with mutations in *mpr*F (multiple peptide resistance factors) and *vra*SR (vancomycin resistance-associated senso-regulator system) genes. Provided evidence shows that upregulation of *vra*SR is a key factor associated with daptomycin resistance and that inactivation results in increased DAP susceptibility. It was also found that *vra*SR is a critical regulator of cell membrane homeostasis in response to the alteration of membrane surface charges and reorganization of cell division proteins associated with cell wall synthesis. Upregulation of *vra*SR leads to an increase in cell wall thickness and limited binding of daptomycin. When the *vra*SR operon is removed from the genome of *S. aureus*, the level of daptomycin resistance decreases and susceptibility is achieved. (20; 42) On the other hand, the *mpr*F gene encodes important membrane protein (which is present in phospholipid synthesis). A mutation on the *mpr*F gene causes a positive charge increase on the cell membrane (i.e. neutralization of the negative charge of the cell membrane), making it difficult for the positively charged daptomycin-calcium complex to bind. (29) As such, the resistance of *S. aureus* to daptomycin is

characterized by enhanced fluidity of the membrane, an increased charge of the membrane surface, lower susceptibility to depolarization induced by daptomycin, and reduced binding ability of daptomycin. (31; 42). Another reason for daptomycin resistance may be due to selective pressure caused by the administration of daptomycin or vancomycin (20; 41). However, once the pressure is removed, previously non-susceptible strain with thickened cell walls will reverse to susceptible strain (31).

- **Indication:** Daptomycin is a suitable choice of antibiotic for SSTI, bacteremia caused by MRSA, endocarditis, or osteomyelitis (22; 28; 31; 46; 47). Daptomycin is also a fitting substitute for vancomycin in cases of resistance to vancomycin (vancomycin MIC needs to be confirmed) (17; 40), intolerance to vancomycin, for patients with impaired kidney function or with a high risk of nephrotoxicity, and failed therapy with vancomycin (26; 44; 45). Daptomycin is contraindicated for pneumonia due to the inactivation of daptomycin by a pulmonary surfactant (22; 28; 41; 45; 48), and should not be used for CNS infections because of its low bioavailability in cerebrospinal fluid (38).

3.4.4. Lipoglycopeptides (dalbavancin, oritavancin, telavancin)

- **Mode of action:** lipoglycopeptides are bactericidal antibiotics with heptapeptide core (typical for glycopeptides), which causes inhibition of transglycosylation and transpeptidation by binding on terminal D-ala-D-ala part of peptidoglycan chain, thus being responsible for impairing cell wall synthesis of bacteria. The additional lipophilic side chain fastens the molecule to the membrane of the cell, thus increasing the efficacy of the antibiotic by increasing its concentration at the site of action. Additionally, lipophilic side chains can also contribute to the destabilization of the cell membrane and loss of its potential (only in oritavancin and telavancin). This dual mechanism of action may contribute to increased effectiveness, rapid activity, and decreased risk of resistance. It is also speculated that oritavancin inhibits RNA synthesis. (29; 38; 49)

- **Mechanism of resistance:** So far, the incidence of lipoglycopeptide resistance is rare. While their long half-life may be the reason for rare occurrences of resistance, it also could cause resistance due to their use at subinhibitory concentrations. Few cases of such dalbavancin resistance were reported. While the resistance could not emerge from MRSA or VISA selection, it could arise from prolonged use of subinhibitory concentrations of dalbavancin or vancomycin. (42) Resistance can also occur by modification of the target site of action, similar to VRSA resistance. Modification of the terminal D-ala-D-ala part of the peptidoglycan chain makes it impossible for telavancin and dalbavancin to bind. This type of resistance does not occur in oritavancin, likely due to its multiple mechanisms of action. All three lipoglycopeptides retain susceptibility against VISA/hVISA. (31)

- **Indication:** lipoglycopeptides show activity against MRSA, VISA, and VRSA, except for dalbavancin being active only against MRSA and VISA (29). They are suitable substitute antibiotics in case of failure of vancomycin or daptomycin therapy. (49)

o **dalbavancin**: owning to its long half-life (147–258 hours), it can be administrated once a week for *S. aureus* infections (38; 42; 44), such as complicated SSTI, catheter-related bacteremia (28; 29; 49), and is suitable for OPAT (17; 26).

o **oritavancin**: this lipoglycopeptide achieves high concentrations in macrophages, which is a useful characteristic given that *S. aureus* infections are often persistent due to *S. aureus* taking resistance inside cells. Oritavancin can be used under the same conditions as dalbavancin, for uncomplicated, catheter-related bacteremia, and complicated SSTI (28; 29; 49), and is suitable for OPAT (17). Given its extremely long half-life (up to 450 h), oritavancin is administrated just once during treatment (38; 42; 49)

o **telavancin**: telavancin's clinical use is mainly as a vancomycin alternative for complicated SSTI, HAP, ventilator-associated pneumonia (VAP), and bacteremia (28; 38; 41; 48; 49). Due to its nephrotoxicity, it should not be used in patients with impaired kidney function (44; 45). Telavancin also has a relatively long half-life (7–9 hours) and is administrated once a day (42).

3.4.5. Oxazolidinones (linezolid, tedizolid)

- **Mode of action:** Oxazolidinones are antibiotics with bacteriostatic effect on *S. aureus*, and inhibit protein synthesis of bacteria by binding to 23S ribosomal RNA (rRNA) of the 50S ribosomal subunit. This prevents the binding of aminoacyl-tRNA in the peptide-transfer center, thus impeding the formation of the 70S initiation complex. (2; 22; 42; 50) Tedizolid has an advantageous modification in its chemical structure that enhances interactions at the binding site, increasing its efficacy (29; 31; 45).

- **Mechanism of resistance (linezolid):** there are three mechanisms of resistance described in oxazolidinones, and all of them alter the binding site:

o Mutation in domain V of the 23S rRNA genes is the most common type of resistance. *S. aureus* carries multiple copies of 23S rRNA, and several mutations determine the potency of linezolid resistance (31; 42; 51). This type of resistance is commonly associated with prolonged use of linezolid (17; 20; 38).

o Resistance caused by mutations of genes encoding L3/L4 ribosomal proteins exhibits a similar effect as above mentioned resistance but is not so common (20; 31; 42; 51).

o Acquisition of chloramphenicol-florfenicol resistance (*cfr*) determinant, which encodes ribosomal methyltransferase, grants resistance to several classes of antibiotics (like lincomycins, macrolides, aminoglycosides, fluoroquinolones, streptogramins, amfenicols), oxazolidinones included (17; 20). The methyltransferase alters the binding site position at the ribosomal peptide-transferase center of the 23S rRNA by methylation, creating a steric obstruction. (42; 51). The transferable *optr*A gene, which often coexists with the *cfr* gene, also causes resistance to oxazolidinones, although its mechanism of action is unknown (31). The *cfr* gene is transferred to MRSA strains through plasmid from other Gram-positive

pathogens (like streptococci, macrococci, bacilli, enterococci, ….) (42). These pathogens are often present in livestock and the food industry, and thanks to the prophylactic use of amphenicols, the selective pressure leads to increased prevalence of the *cfr* gene. This type of resistance is common in patients without previous exposure to linezolid and may be hard to battle with. (51)

- The occurrence of resistance to oxazolidinones is quite rare. Thanks to its modified chemical structure, tedizolid remains unaffected by resistance from mutation of genes encoding L3/L4 ribosomal proteins, and *cfr* gene type of resistance. However, if an additional *optr*A gene is present, even tedizolid treatment may be ineffective. (29; 31; 42; 51)

- **Indication:**

- **Linezolid** is the first-choice alternative to vancomycin. It is suitable for the treatment of MRSA infections like SSTI, HAP/CAP, and diabetic foot infections without osteomyelitis, due to its extensive penetration into tissues (17; 22; 28; 44; 45; 48; 52).

- **Tedizolid** is used against SSTI and linezolid-resistant MRSA infection (17; 38; 45; 48).

- Oxazolidinones are unfit for infective endocarditis and bacteremia (40).

3.4.6. Fluoroquinolones (ciprofloxacin, delafloxacin)

- **Mode of action:** fluoroquinolones are bactericidal antibiotics that block the replication of bacterial DNA. This antibiotic class has a dual target of action— topoisomerase II (DNA gyrase) and topoisomerase IV. (2; 41; 50) Quinolones can differ in their potency for the two enzymes, with a general pattern among quinolones in clinical use that there is greater activity against DNA gyrase in Gram-negative bacteria and greater activity against topoisomerase IV in Gram-positive bacteria; but exceptions occur, and some quinolones have similar potency against both enzymes (42; 50). The break of the DNA double-strand, brought on by the inhibition of either or both enzymes, is the reason for the cell's death. Delafloxacin, a novel fluoroquinolone, has a structural modification, which increases its efficacy and spectrum of activity. (31; 53)

- **Mechanism of resistance:** resistance to fluoroquinolones can be caused by the combination of two types of mechanisms:

- <u>Mutation of genes encoding target enzymes:</u> for DNA gyrase, it is a mutation on genes *gyr*A/*gyr*B; for topoisomerase IV, it is a mutation on gene *grl*A/*grl*B. While both mutations can cause resistance separately, the combination of both accounts for a higher efficacy of resistance.

- <u>Overexpression of efflux pumps:</u> *S. aureus* demonstrates three types of efflux pump systems *(Nor, Mde, Qac),* and their affinity to fluoroquinolone agents varies— for example, *Nor*A is responsible for ciprofloxacin resistance.

- o After the introduction of ciprofloxacin, resistance to fluoroquinolones has rapidly accelerated in *S. aureus*, especially in MRSA. (2) This fact has been associated with the overuse of fluoroquinolones and inappropriate drug dosing (especially in hospitals) (41; 54).

- o Delafloxacin is not included on the list of antibiotics with fluoroquinolone resistance. This phenomenon is credited to the specific chemical structure of delafloxacin, and the accumulation of several mutations is needed for resistance manifestation. (2; 31; 42; 53)

- **Indication:** although older fluoroquinolones (like ciprofloxacin and levofloxacin) retained their effectiveness against CA-MRSA infection such as infective endocarditis (44), they are not recommended for monotherapy due to their prevalent resistance (31; 42).

- o **Delafloxacine** is the only fluoroquinolone approved for the monotherapy of MRSA SSTI (28; 53; 55), and thanks to its chemical structure and low potential for mutation resistance, it can be used against quinolone-resistant MRSA, and biofilm-producing MRSA. (17)

3.4.7. Tetracyclines/glycylcyclines (tigecycline, omadacycline)

- **Mode of action:** Tetracyclines exhibit bacteriostatic effects on *S. aureus*. They inhibit protein synthesis by binding onto the 30S ribosomal subunit (2; 56). Tigecycline, a minocycline analog, is a member of glycylcyclines, a new class of tetracyclines (57). Tigecycline inhibits protein translation and blocks the entry of the aminoacyl part of tRNA into the a side of the 30S ribosome (29; 42). Omadacycline is an aminomethylcycline derivative (31).

- **Mechanism of resistance:** Tetracycline resistance is associated with *tet* and *ort* genes. The *tet* gene plays a more significant role in tetracycline resistance. Different types of *tet* gene are recognized to encode different mechanisms of tetracycline resistance:

- o *tet*A/K/L genes cause active efflux of tetracycline compounds

- o *tetM/O* genes are responsible for protecting the target site of tetracycline on the bacterial ribosome. (2; 42)

- o Tigecycline structural modification was specifically made to battle tetracycline resistance. The modified side chain induces steric hindrance and protects the tigecycline from efflux protein pumps. (17; 29; 58) However, MRSA has developed resistance to tigecycline by mutations in the efflux pump *Mep*A and in the transcriptional regulator *Mep*R (31; 42).

- o The chemical structure of omadacycline was developed to combat tetracycline resistance. Omadacycline is not influenced by any *tet* genes (44).

- **Indication:**

- o **Tigecycline**: low serum concentrations are achieved in tigecycline treatment, which is not suitable for the treatment of MRSA bacteremia (17; 29; 38; 45). It can be used for complicated SSTI, and off-label for intra-abdominal infections, pneumonia, and diabetic

foot infections caused by MRSA; however, tigecycline is considered a third-choice drug (28; 48; 58).

- o **Omadacycline**: this drug is approved for SSTI and CAP caused by MRSA (28; 44; 58).

3.4.8. Rifampicin

- **Mode of action:** Rifampicin is a bactericidal antibiotic drug that inhibits RNA synthesis by binding to the β-subunit of DNA-dependent RNA polymerases. This mechanism inhibits bacterial transcription, subsequently leading to the cell death. The β-subunit is encoded by the *rpo*B gene. Rifampicin can also penetrate microbial biofilms. (41; 42; 50; 56)

- **Mechanism of resistance:** Resistance to rifampicin occurs through the *rpo*B gene mutation (42).

- **Indication:** Rifampicin is not recommended for monotherapy of MRSA infections due to the high prevalence of rifampicin resistance (48). However, rifampicin has shown to be a rather favorable agent for combination therapy, thanks to its activity against biofilms, and good tissue and intracellular penetration (25; 26; 46; 57; 59; 60). Combination antimicrobial therapy with rifampicin can be used for deep tissue, joint, and bone infections, and infections related to implanted medical devices; but is not recommended for *S. aureus* infections that do not originate from prosthetics (39; 41; 56; 61; 62).

3.4.9. Trimethoprim-sulfamethoxazole (cotrimoxazole)

- **Mode of action:** Cotrimoxazole is a combination of two antimicrobial agents that both target sequential steps in the synthesis of folic acid (an important cofactor in the synthesis of amino acids and nucleotides) in bacteria. Trimethoprim inhibits dihydrofolate reductase (DHFR) responsible for the catalysis of dihydrofolate (DHF) to tetrahydrofolate (THF) and has a stronger affinity to bacterial DHFR compared to its human counterpart. Sulfamethoxazole inhibits dihydropteroate synthase (DHPS), an enzyme responsible for coupling pteroate with para-aminobenzoic acid (PABA) to create dihydropteroate (DHP). (42; 50)

- **Mechanism of resistance:** Resistance to cotrimoxazole occurs through chromosomal mutations— for sulfamethoxazole, it is on the gene that encodes DHPS, reducing the affinity of sulfamethoxazole to the target binding site; for trimethoprim, the mutation of the *dfr*B gene, which encodes DHFR, and the acquisition of different *dfr* genes (*dfr*A/G/K), is the cause of resistance. (2; 42; 50)

- **Indication:** Cotrimoxazole is not a suitable option for severe infections (bacteremia or abscesses) (17), but can be used in monotherapy for uncomplicated SSTI or osteomyelitis (28). Cotrimoxazole can be used in combination with vancomycin or daptomycin for the treatment of difficult-to-treat MRSA infections such as persistent MRSA bacteremia (29; 39), and with rifampicin for deep tissue, joint, and bone infections (56).
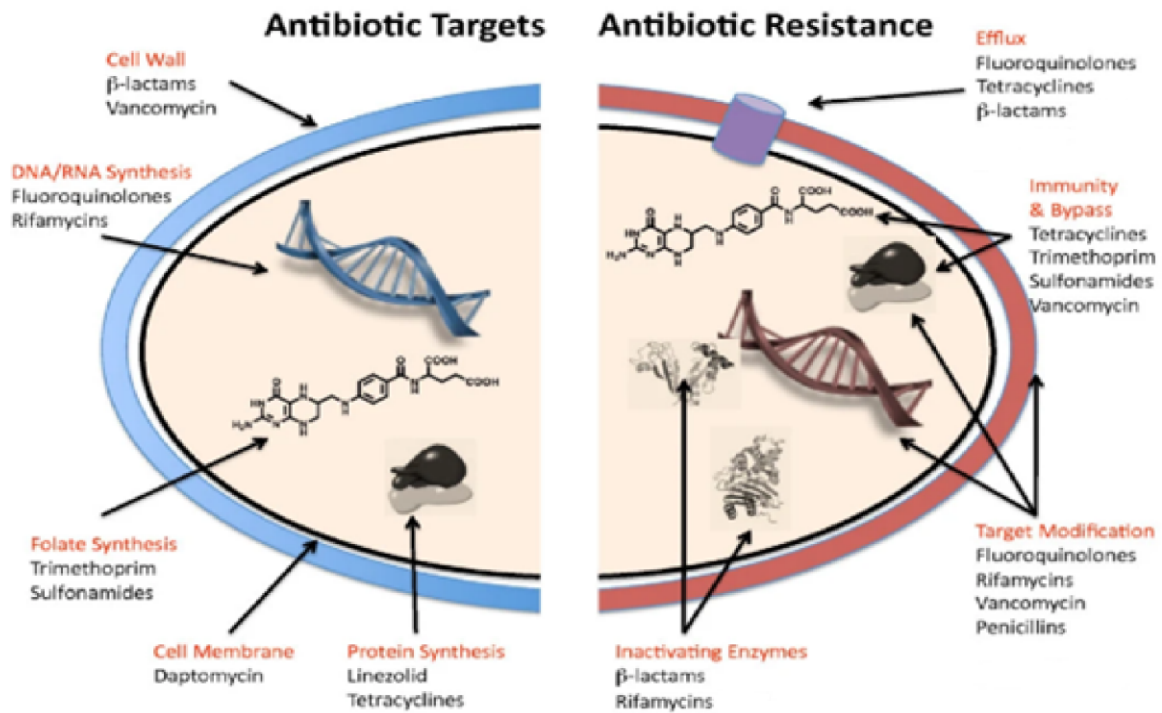
Figure 5: *Schematic representation of antibiotic classes, their targets in the microbial cells, and mechanisms of resistance (edited). Source: Ahmad, et al.; Characterization of novel antibiotic resistance genes in Staphylococcal aureus; 2018* (54)

3.5. Combination antimicrobial therapy

As mentioned above, the use of antibiotics in the treatment of MRSA infections can have some limitations. Vancomycin has duration-dependent nephrotoxicity and variable tissue penetration, and so-called MIC "creep" for vancomycin was registered. MIC "creep" is a controversial phenomenon that describes the gradual increase of vancomycin MIC that went unnoticed for some time, resulting in lower efficacy of vancomycin treatment, even though *S. aureus* isolate was still considered vancomycin-susceptible, with vancomycin MIC being on the upper end of susceptibility range (MIC= 2mg/L). (23; 28; 38; 41; 44) This phenomenon is associated with excessively long administration of vancomycin at sub-optimal concentrations. However, the occurrence and level of "creep" appear to be variable depending on the region and frequency of vancomycin use in that particular region. (63) Daptomycin cannot be used for pulmonary infections, and the cost of linezolid therapy is higher (39; 57). As for newer antimicrobials such as lipoglycopeptides, teicoplanin, or omadacycline, they are seldomly used and are kept as reserved for exceptional cases like treatment of MDR/XDR infections, and their usage should be approved by the specialist on infectious diseases and clinical pharmacist. (58)

Infections caused by MRSA have a two-times higher mortality rate than MSSA infections. While there are plenty of antibiotic treatment options for MSSA infections and uncomplicated MRSA infections, more serious, invasive MRSA infections, as well as infections caused by VISA, hVISA, VRSA, and DNSA pose a real challenge for a successful treatment strategy. (39; 64) Since antibiotic monotherapy of infections caused by MDR *S. aureus* strains is rather limited, combination antimicrobial therapy seems a promising alternative. There are some advantages of combination therapy— it is a strategy to avoid or limit resistance development, to broaden the spectrum of effect, and to reduce doses of antibiotics, which in turn may reduce their toxicity and improve clinical efficacy. (65; 66) The key to rational combination therapy is the employment of drugs in combinations with synergic antimicrobial effects and low human cell toxicity. The first step for the recognition of suitable and desired combinations is to determine the activity of antibiotic drugs in combinations *in vitro*. There are several methodical approaches for the study of the impact of the combination on antimicrobial action: agar diffusion method, checkerboard assay, study of time-kill curves, and simulated pharmacodynamic models. Antibiotic drugs in combinations can express synergistic drug interaction, indifferent or antagonistic interaction. (57; 65) The ideal combination of antibiotics should demonstrate sufficient inhibition of bacterial growth at subinhibitory concentrations and synergy *in vitro* (67).

4. EXPERIMENTAL PART

4.1. Materials and methods

4.1.1. Bacterial strain

For testing the antibacterial activity of two approved antibiotic drugs in combination, the methicillin-resistant strain of *Staphylococcus aureus,* MRSA, American Type Culture Collection, ATCC 43300, CCM 4750, purchased from the Czech Collection of Microorganisms (CCM) was employed. Bacterial suspension in cation-adjusted Müller-Hinton broth (CAMHB) with a density corresponding to 0.5 McFarland units, was prepared from the overnight bacterial culture grown on Müller-Hinton agar.

4.1.2. Chemicals

- Dimethylsulfoxide (DMSO) (Merck, Steinheim, Germany)

- Cation-adjusted Müller-Hinton broth (CAMBH) (Merck, Steinheim, Germany)

4.1.3. Antibiotics

Seven commercially available antibiotics were employed in seventeen pairwise combinations (see Table 4).

- Vancomycin (Merck, Steinheim, Germany)

- Daptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

- Linezolid (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

- Ciprofloxacin (Merck, Steinheim, Germany)

- Tigecycline (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

- Cotrimoxazole (Cayman Chemical, Ann Arbor, Michigan, USA)

- Rifampicin (Merck, Steinheim, Germany)

4.1.4. List of materials and laboratory equipment

- One-channel micropipettes Eppendorf, volume 1–50 μl, 2–200 μl, 0.5–5 mL (Eppendorf, Hamburg, Germany)

- Multi-channel micropipette Eppendorf, volume 10–100 μl (Eppendorf, Hamburg, Germany)

- Sterile plastic tips for micropipettes Eppendorf (Eppendorf, Hamburg, Germany)

- PP test tube GAMA (Gamedium, Jesenice, Czech Republic)

- Eppendorf safe-lock microtube, volume size 1.5 ml (Eppendorf, Hamburg, Germany)

- Test tube rack and micro test tube rack (Brandt, Wertheim, Germany)

- Microtitre plates with lids GAMA (Gamedium, Jesenice, Czech Republic)

- Foils for microtitre plates (VWR International, Radnor, Pennsylvania USA)

- Laboratory Liquid Transfer Troughs (Brandt, Wertheim, Germany)

- Laminar Box ESCO (Esco Micro Pte. Ltd., Singapore)

- Analytical balances Mettler (Mettler-Toledo, Columbus, Ohio, USA)

- Thermostat Binder (Binder, Tuttlingen, Germany)

- The BioTek Synergy HTX Multi-Mode Microplate reader (Agilent, Santa Clara, California, USA)

4.1.5. Checkerboard microdilution method

In vitro antimicrobial susceptibility testing is a standard method to predict the response of a tested microorganism to exposure to an antimicrobial compound *in vivo*, helping to select the most appropriate one. There are various applicable methods such as the disk-diffusion method, the antimicrobial gradient method (E-test), the dilution methods (micro- or macro-dilution), or the time-kill test. Broth microdilution is the reference method for fast-growing aerobic bacteria. The determined activity of antimicrobial agents is expressed in minimum inhibitory concentration (MIC) values. Two-fold serial dilution is used to test two antibiotic agents in a checkerboard assay, testing two agents both in combination and alone. Varying concentrations of two antibiotics can be dispensed along the columns and rows to allow for the determination of MIC for each antibiotic in combination. This makes it possible to determine the efficacy of each drug and the effect of the tested combination. The factors influencing MIC values are the inoculum size, the type of growth medium, the incubation time, and the inoculum preparation method.

The Clinical Microbiology Procedure Handbook 3rd Edition defines the high-throughput method, where a 96-well microplate is used, as the most appropriate and consolidated, although time-consuming method. (57; 68; 69; 70)

4.1.6. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial agent that inhibits the bacterial growth of the tested microorganism after some incubation period. The value of MIC can be expressed in mg/L (µg/mL) or µM. MIC can be used to confirm the emergence of resistance to pathogens or to determine *in vitro* efficacy of tested antimicrobial agents. MIC can be determined by more methodical approaches:

- dilution methods (in agar or liquid medium/broth): micro-method (micro-dilution) or macro-method (macro-dilution)

- gradient methods: E-test (strip is infused with a defined concentration gradient of antiinfective drug). Both methodical approaches use Müller-Hinton as the medium for determining MIC values of drugs acting against bacteria, either in agar (MHA) or broth (MHB). A medium can be supplemented with additional components. (71; 72)

In this thesis, the microdilution broth method was used to assess the efficacy of antibiotic combinations.

4.1.7. Determination of minimum inhibitory concentration

In Table 1, the EUCAST MIC breakpoints of selected antibiotics are illustrated (version 14.0, 1 Jan 2024 (73)). MIC values of selected antibiotics for MRSA strain ATCC 43300 determined within preliminary evaluation are present in Table 2. The MIC values of selected antibiotics were also re-determined within each checkerboard assay (intra-assay evaluation), and are stated in Table 3. In Table 4 are listed pair-wise combinations of antibiotics tested in this thesis.

Table 1: *Minimum inhibitory concentrations (MIC) breakpoints of selected antibiotics for strains Staphylococcus spp. Values were obtained from EUCAST,* [v_14.0_Breakpoint_Tables.pdf (eucast.org)](#)*, pages 32-38.* (73)

Drug	MIC breakpoint for *S. aureus* (mg/L)	
	susceptible	resistant
vancomycin	≤ 2	> 2
daptomycin	≤ 1	> 1
linezolid	≤ 4	> 4
ciprofloxacin	≤ 0.001	> 2
tigecycline	≤ 0.5	> 0.5
cotrimoxazole	≤ 2	> 4
rifampicin	≤ 0.06	> 0.06

Table 2: *Determined minimum inhibitory concentration (MIC) values for selected antibiotics acting against methicillin-resistant Staphylococcus aureus (MRSA) ATCC 43300.*

Drug	Determined MIC breakpoint for MRSA ATCC 43300 (mg/L)
vancomycin	1
daptomycin	2
linezolide	8
ciprofloxacin	0.25-0.5
tigecycline	0.0625
cotrimoxazole	2
rifampicin	0.005

Table 3: *Re-determined minimum inhibitory concentration (MIC) values for selected antibiotics acting against methicillin-resistant Staphylococcus aureus (MRSA) ATCC 43300 within checkerboard assays, and classification of their susceptibility according to EUCAST breakpoint values in Table 1. Legend: S= susceptible, R= resistant, I= susceptible, increased exposure, ND= not determined.*

| Drug	Re-determined MIC breakpoint for MRSA ATCC 43300 (mg/L)	Classification
vancomycin	1–2	S
daptomycin	>8	R
linezolid	2–4	S
ciprofloxacin	0.25–0.5	I
tigecycline	>0.25	ND*
cotrimoxazole	2	S
rifampicin	0.005–0.01	S

*the tested concentration range must be adapted

Table 4: *List of tested pair-wise antibiotic drug combinations. Legend: VAN= vancomycin, DAP= daptomycin, LIN= linezolid, CIP= ciprofloxacin, TIG= tigecycline, COT= cotrimoxazole, RIF= rifampicin*

1. antibiotic	2. antibiotic
VAN	DAP
	LIN
	CIP
	TIG
	COT
	RIF
DAP	LIN
	CIP
	RIF
LIN	CIP
	TIG
	COT
	RIF
CIP	TIG
	COT
COT	RIF

4.1.8. Preparation of stock solutions for selected antibiotic drugs

First, a small amount (in mg) of antibiotic drug was put into an Eppendorf safe-lock tube from the vial of a commercially purchased antibiotic agent. DMSO was used as the solvent. The drug solved in DMSO was serially diluted by a two-fold serial dilution method. Finally, 20 μL of the selected drug dissolved in DMSO was transferred into 1980 μL of CAMBH. The final concentration of DMSO corresponded to 1% v/v.

The workflow of two-fold dilution is to take the pre-determined volume of antibiotic solution and dilute it with the same amount of solvent — in this thesis, the amount of drawn-out antibiotic solution and amount of solvent was 100 μl. The next step is to take 100 μl of diluted antibiotic solution and dilute it again with 100 μl of solvent. Repeat this action till the desired concentration range is achieved (see Figure 6).
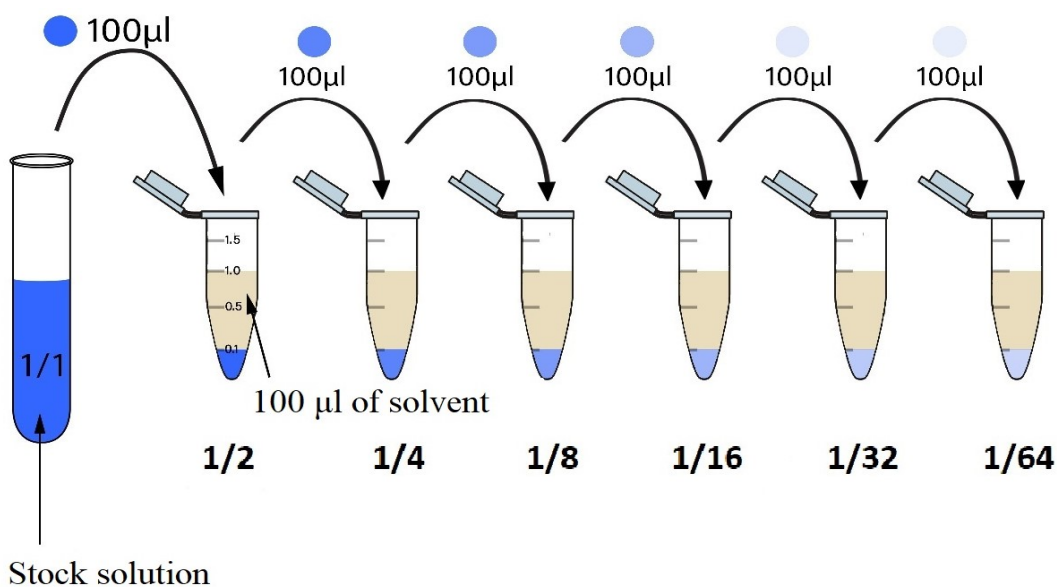
Figure 6: *Schematic representation of two-fold serial dilution (original web template taken from* https://www.medicine.mcgill.ca/physio/vlab/Vlab_in_progress/dilutions.html, *and edited).*

The next paragraphs describe the solution preparation workflow for each antibiotic agent for checkerboard assays:

- for LIN and VAN

  o weight 5 mg and dissolve in 0.5 ml (500 μl) DMSO → stock solution with concentration 10 mg/ml (10 000 mg/L) is reached

  o the required primary concentration is 1600 mg/L (16 mg/L X 100) → take 160 μl of stock solution and add 840 μl of DMSO

  o apply method of two-fold serial dilution → the desired concentration range 1600–800–400–200–100–50–25–12.5–6.25–3.125... mg/L

  o for final dilution, use CAMHB. Apply two-fold serial dilution with a 1:100 ratio → take 20 μl of intermediate solution and 1980 μl of broth. Repeat with every concentration → final concentration line: 16–8–4–2–1–0.5–0.25–0.125... mg/L

- for DAP

  o weight 5 mg and dissolve in 0.5 ml (500 μl) DMSO → stock solution with concentration 10 mg/ml (10 000 mg/L)

  o the required primary concentration is 800 mg/L → take 80 μl of stock solution and dilute with 920 μl DMSO

  o apply method of two-fold serial dilution → the desired intermediate concentration line is 800–400–200–100–50–25–12.5–6.25... mg/L

  o for final dilution, use CAMHB. Apply two-fold serial dilution with a 1:100 ratio → take 20 μl of intermediate solution and 1980 μl of broth. Repeat with every concentration → final concentration line: 8–4–2–1–0.5–0.25–0.125–0.0625... mg/L

- for CIP

  o weight 5 mg and dissolve in 0.5 ml (500 μl) DMSO → stock solution with concentration 10 mg/ml (10 000 mg/L)

  o the required primary concentration is 100 mg/L → take 10 μl of stock solution and dilute with 990 μl DMSO

  o apply the method of two-fold serial dilution → the desired intermediate concentration line is 100–50–25–12.5–6.25–3.125–1.5625–0.078125... mg/L

  o for final dilution, use CAMHB. Apply two-fold serial dilution with a 1:100 ratio → take 20 μl of intermediate solution and 1980 μl of broth. Repeat with every concentration → final concentration line: 1–0.5–0.25–0.125–0.0625–0.03125–0.015625–0.0078125... mg/L

- for TIG

  o weight 5 mg and dissolve in 0.5 ml (500 μl) DMSO → stock solution with concentration 10 mg/ml (10 000 mg/L)

  o the required primary concentration is 25 mg/L → take 2.5 μl of stock solution and dilute with 997.5 μl DMSO

  o apply the method of two-fold serial dilution → the desired intermediate concentration line is 25–12.5–6.25–3.125–1.5625–0.078125–0.0390625–0.01953125... mg/L

  o for final dilution, use Müller-Hinton broth. Apply two-fold serial dilution with a 1:100 ratio → take 20 μl of intermediate solution and 1980 μl of broth. Repeat with every concentration → final concentration line: 0.25–0.125–0.0625–0.03125–0.015625–0.0078125–0.00390625–0.001953125... mg/L

- for COT

  o weight 5 mg and dissolve in 0.5 ml (500 μl) DMSO → stock solution with concentration 10 mg/ml (10 000 mg/L)

  o the required primary concentration is 400 mg/L → take 40 μl of stock solution and dilute with 960 μl DMSO

  o apply the method of two-fold serial dilution → the desired intermediate concentration line is 400-200-100-50-25-12.5-6.25-3.125... mg/L

  o for final dilution, use Müller-Hinton broth. Apply two-fold serial dilution with a 1:100 ratio → take 20 μl of intermediate solution and 1980 μl of broth. Repeat with every concentration → final concentration line: 4–2–1–0.5–0.25–0.125–0.0625–0.03125... mg/L

- for RIF

  o weight up 5 mg and dissolve in 1 ml (1000 μl) DMSO → concentration is 5 mg/ml (5 000 mg/L), dilute 10× → take 100 μl of solution and 900 μl DMSO → stock solution with concentration 500 mg/L.

  o the required primary concentration is 4 mg/L → take 8 μl of stock solution and dilute with 992 μl DMSO

  o apply the method of two-fold serial dilution → the desired intermediate concentration line is 4–2–1–0.5–0.25–0.125–0.0625–0.03125–0.0156–0.00781... mg/L

  o for final dilution, use CAMHB. Apply two-fold serial dilution with a 1:100 ratio → take 20 μl of intermediate solution and 1980 μl of broth. Repeat with every concentration → final concentration line is 0.04–0.02–0.01–0.005–0.0025–0.00125–0.000625–0.0003125–0.000156–0.0000781... mg/L

4.1.9.   Pipetting of antibiotic and bacterial solutions using the checkerboard method

Within the high-throughput approach, one 96-well microtitre plate was used for two independent checkerboard assays (two pair-wise combinations) simultaneously. Solutions of antibiotics in CAMBH were pipetted in a two-fold decreasing manner— the first agent was pipetted vertically, the second one horizontally, per a pre-determined pipetting scheme. Two lines were reserved for the evaluation of the antibacterial activity of each antibiotic acting alone (internal evaluation of MIC value for selected drug(s)), and one line was used for pipetting of suspension of bacteria not exposed by any antibiotic drug (positive control) (see Figure 7).

It is important to note that the final concentration of each antibiotic drug in combination, transferred into wells of microtitre plates, corresponds to the half value of the concentration of drug in CAMBH solution (e.g. if the solution of antibiotic drug in CAMBH corresponds to 4 mg/L, the final concentration in the well corresponds to 2 mg/L.

The workflow was as follows:

- for drug combinations – add to wells 100 µl of drug A and drug B solution in CAMHB according to the pre-prepared scheme (Figure 7)

- for drug acting alone – add 200 µl of each drug A and drug B solution in CAMHB in wells of microtitre line according to the scheme (Figure 7)

- for the positive control (PC) – add 200 µl of CAMHB in wells of the line designated by shortcut PC (Figure 7)

- inoculate every well with 10 µl of bacterial suspension

- carry out incubation for 24 hours at a temperature of 37°C

The final concentration of VAN in checkerboard assays ranged from 2 mg/L to 0.0625 mg/L. The final concentration of DAP corresponded to the range of 4 mg/L to 0.125 mg/L. The final concentration of LIN ranged from 8 mg/L to 0.25 mg/L. The final concentration of CIP ranged from 0.5 mg/L to 0.0625 mg/L, for TIG, the final concentration ranged from 0.0625 mg/L to 0.001953125 mg/L. The final concentration of COT ranged from 1 mg/L to 0.03125 mg/L. And lastly, the concentration of RIF ranged from 0.01 mg/L to 0.0003125 mg/L.

The concentration of VAN alone, as well as the concentration of DAP alone, ranged from 8 mg/L to 0.25 mg/L. The concentration range of LIN alone was set from 16 mg/L to 0.5 mg/L, and for CIP alone, the concentrations ranged from 1 mg/L to 0.03125 mg/L. The concentration range of TIG alone was from 0.25 mg/L to 0.0078125 mg/L. The concentration of COT alone corresponded to the range of 4 mg/L to 0.125 mg/L, and the concentration of RIF alone ranged from 0.02 mg/L to 0.000625 mg/L.

ATB A →	Cf (mg/L)	1/2X	1/4X	1/8X	1/16X	1/32X	1/64X
ATB B ↓	Cp (mg/L)	X	1/2X	1/4X	1/8X	1/16X	1/32X

Cf (mg/L)	Cp (mg/L)		1	2	3	4	5	6	7	8	9	10	11	12	
1/2X	X	A													
1/4X	1/2X	B													
1/8X	1/4X	C													
1/16X	1/8X	D													
1/32X	1/16X	E													
1/64X	1/32X	F													
ATB A Alone		G													ATB B Alone
PC		H													

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Figure 7: *Template for pipetting scheme (original web template taken from https://www.cellsignet.com/media/templ.html) and edited). Legend: C_p = pipetted concentration of antibiotic drug A/drug B, C_f = final concentration of antibiotic drug A/drug B, PC= positive control*

4.2. Evaluation of the antibiotic action efficiency of drugs in combination by spectrophotometric measurement and visual inspection

Spectrophotometric measurement is a method based on measuring the light transmitted or absorbed through a sample. Turbidity indicates the presence of small insoluble particles in suspension, creating cloudiness or haziness. For bacterial growth assays, microplate readers for measuring the transmission of light through the sample are mostly employed. The optical density (or the measurement of the absorbance) that bacterial growth generates by scattering the light is the measurement of turbidity. The more turbid the suspension is, the less amount of light is transmitted. This fact can be used to calculate the degree of turbidity and absorbance values can be used to calculate the percentage of inhibition. (74)

To confirm the lack of bacterial growth, a visual inspection was also conducted. If a well displayed any kind of turbidity to the naked eye, it was considered that the antibiotic inhibition was not sufficient enough.

### 4.3. Data evaluation and interpretation of results

4.3.1. Calculation of the percentage of inhibition and creation of heat maps

Absorbance values acquired from the spectrophotometric evaluation were used to calculate the percentage of growth inhibition for each well. From the optical density, it is possible to calculate the percentage of growth for each well.

$$percentage\ of\ inhibition\ (\%) = \left(\frac{A_{Pk} - A_x}{A_{Pk}}\right) \times 100$$

Figure 8: *Equation for calculating the percentage of growth inhibition. Legend: A_{PK}= average of absorbance values for suspensions with positive control(s) (microorganisms unexposed to drug), A_X= absorbance of bacterial suspensions exposed to drug alone or drugs in combination*

Results from checkerboard assays can be represented as heat maps. Heat maps are visual representations of acquired data, where a degree of inhibition is depicted by color scale— this data presentation facilitates visualization and the meaning of processed data. The graduated color scale goes from the darkest color (for the lowest percentage of inhibition) to the lightest color (for the greatest percentage of inhibition).

| <0% | 0-10% | 10-20% | 20-30% | 30-40% | 40-50% | 50-60% | 60-70% | 70-80% | 80-90% | 90-95% | 95-100% |
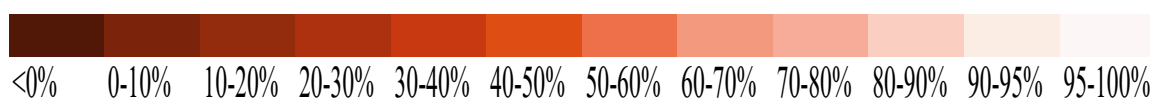
Figure 9: *Graduated color scale for the presentation of data from checkerboard studies within heat maps*

4.3.2. Evaluation of the effect of two selected antibacterial drugs in combination

The fractional inhibitory concentration (FIC) index value was used to determine the interaction of two drugs in combination. The interaction is categorized according to the FIC index value, as illustrated in Table 5.

FIC is calculated by the following formula:

$$\frac{A}{MIC_A} + \frac{B}{MIC_B} = FIC_A + FIC_B = FIC\ Index$$

Figure 10: *Equation for fractional inhibitory concentration (FIC) index calculation. Legend: A= minimum inhibitory concentration of antibiotic drug A in combination, B= minimum inhibitory concentration of antibiotic drug B in combination, MIC_A = minimum inhibitory concentration of antibiotic drug A acting individually, MIC_B = minimum inhibitory concentration of antibiotic drug B acting individually*

Table 5: *Interpretation of fractional inhibitory concentration (FIC) index value. Source: Doern; When Does 2 Plus 2 Equal 5? A Review of Antimicrobial Synergy Testing; 2014.* (75)

FIC Value	Interpretation
≤0.5	Synergy
>0.5-1	Additive
1-4	Indifference
>4	Antagonism

For calculation of FIC values, wells with the lowest concentration of drugs in combination, where the inhibition of the growth was detected, were used (i.e. wells directly above or next to wells with detected bacterial growth).

Note: In the evaluation of FIC index values, and subsequent categorization of mutual interaction of two drugs in combination, the statistical analysis is not performed – see available sources (published studies) mentioned in this thesis, in section Discussion (Chapter 6).

5. RESULTS

5.1. Evaluation of the efficacy of vancomycin and linezolid in combination

Within the high-throughput arrangement of our checkerboard assays, for every drug-drug combination, 6×6 different sub-combinations have been evaluated.

For the preparation of VAN+LIN drug combinations, solutions with different concentrations of VAN in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), solutions of LIN were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. Subsequently, 10 ml per well of the bacterial inoculum was inoculated into wells.

After 24 hours of incubation at 37°C, an evaluation of the inhibition of growth by the naked eye was done. Subsequently, absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as $MIC_{(VAN)}$= 1 mg/L, and $MIC_{(LIN)}$= 4 mg/L. $MIC_{(VAN)}$ within the checkerboard assay corresponded to the same value as determined in Chapter 4.1, while the $MIC_{(LIN)}$ shifted from 8 mg/L to 4 mg/L (see Table 2). According to the EUCATS breakpoints, the MRSA strain is considered susceptible to both antibiotics (see Table 1).

Absorbance values were used to calculate the percentage of growth inhibition using the equation in Figure 8. Finally, all data from the study of the efficacy of VAN+LIN in combination were present as the heat map (see Figure 11).
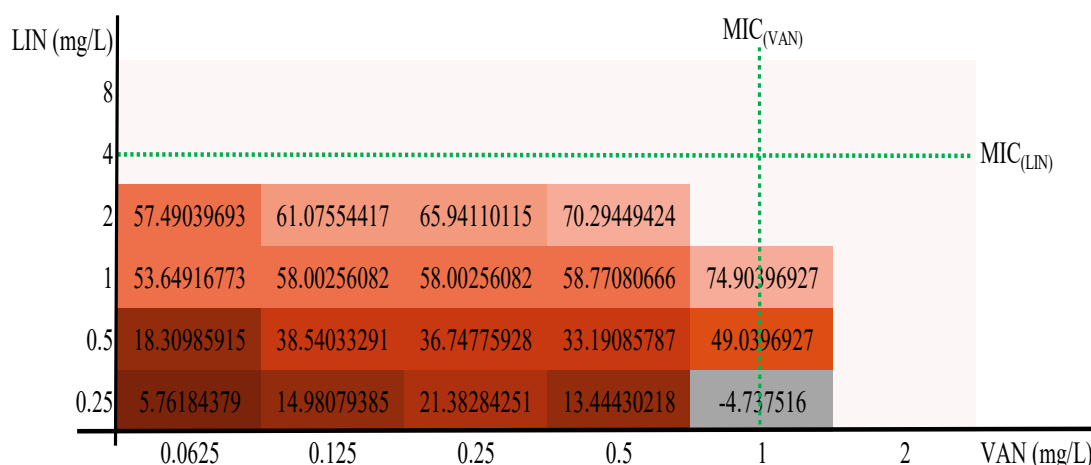
Figure 11*: Heat map of checkerboard assay of vancomycin and linezolid in combination. Heat plot describing the antibacterial activity of vancomycin and linezolid acting in combination against methicillin-resistant Staphylococcus aureus, ATCC 43300 strain. Boxes with values represent the percentage of growth inhibition. Boxes with no values represent wells without the presence of bacterial growth. Boxes in grey were not taken into consideration for evaluation (an error occurred during preparation). Green dotted lines describe the MIC of individual antibiotics. Legend: $MIC_{(VAN)}$= 1 mg/L, $MIC_{(LIN)}$= 4 mg/L, LIN= linezolid, VAN= vancomycin, $MIC_{(LIN)}$= minimum inhibitory concentration of linezolid, $MIC_{(VAN)}$= minimum inhibitory concentration of vancomycin*

All combinations of VAN+LIN with ½ and ¼ MIC sub-inhibitory concentrations of LIN (2 mg/L and 1 mg/L respectively) resulted in more than 50% inhibition of the bacterial growth. Nevertheless, in one combination (VAN:LIN, 1:0.25 mg/L) with the concentration of VAN corresponding to the MIC_{VAN} acting alone (1 mg/L), the inhibition of the growth was not registered.

For the determination of the kind of mutual interaction, the FIC index was calculated according to the equation in Figure 10. Eight concentration ratios, where the lowest MIC was detected, were included in the evaluation (see Table 6). All categorized combinations indicated indifferent effect. Only one combination of VAN+LIN, with a concentration ratio VAN:LIN corresponding to 0.0625:4 mg/L has the FIC index value close to 1, near to additive effect.

Table 6: *Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of vancomycin and linezolid. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, LIN= linezolid, VAN= vancomycin, FIC(A)= fractional inhibitory concentration of vancomycin, FIC(B)= fractional inhibitory concentration of linezolid.*

MIC (VAN : LIN) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
0.5 : 4	1.5	indifference
0.25 : 4	1.25	indifference
0.125 : 4	1.125	indifference
0.0625 : 4	1.0625	indifference, near to additive effect
1 : 2	1.5	indifference
2 : 1	2.25	indifference
2 : 0.5	2.125	indifference
2 : 0.25	2.0625	indifference

5.2. Evaluation of the efficacy of vancomycin and ciprofloxacin in combination

For the preparation of VAN+CIP drug combinations, solutions with different concentrations of VAN in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), and solutions of CIP were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. Subsequently, 10 ml per well of the bacterial inoculum was inoculated into wells.

After 24 hours of incubation at 37°C, an evaluation of the inhibition of growth by the naked eye was done. Subsequently, absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as $MIC_{(VAN)}= 1$ mg/L and $MIC_{(CIP)}= 0.5$ mg/L. MIC of CIP and VAN within the checkerboard assay corresponded to the same value as determined in Chapter 4.1 (see Table 2). According to the EUCAST breakpoints, MRSA strain is recognized as susceptible to both included antibiotics (see Table 1).

Absorbance values were used to calculate the percentage of growth inhibition using the equation in Figure 8, and acquired data were processed into a heat map (Figure 12).
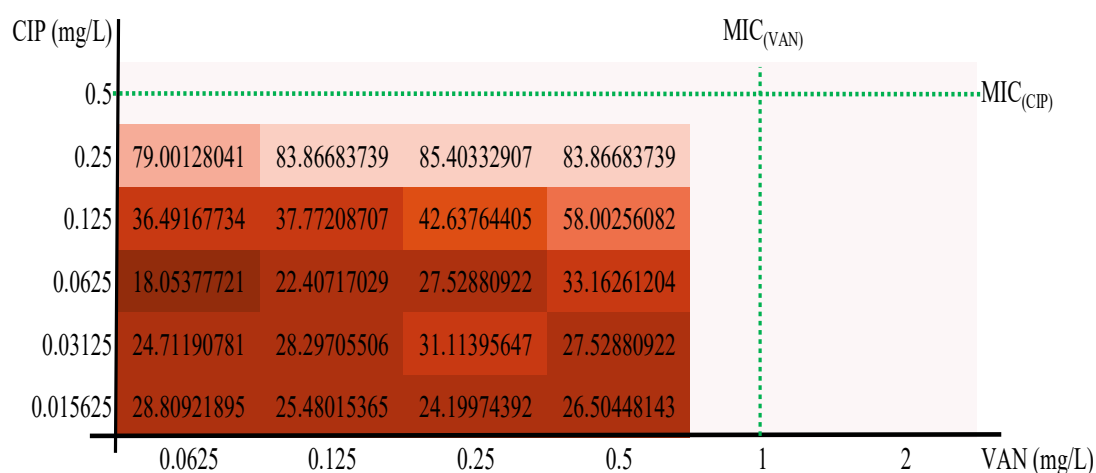
Figure 12*: Heat map of checkerboard assay of vancomycin and ciprofloxacin combination. Heat plot describing the antibacterial activity of vancomycin and ciprofloxacin in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(VAN)}= 1$ mg/L, $MIC_{(CIP)}= 0.5$ mg/L, CIP= ciprofloxacin, VAN= vancomycin, $MIC_{(CIP)}=$ minimum inhibitory concentration of ciprofloxacin, $MIC_{(VAN)}=$ minimum inhibitory concentration of vancomycin*

Figure 12 shows in all combinations of VAN+CIP with ½ MIC sub-inhibitory concentrations of CIP (0.25 mg/L) more than 50% bacterial growth inhibition was achieved. However, in the case of combinations of VAN+CIP with MIC sub-inhibitory concentrations of VAN, the above 50% inhibition occurred only if the concentration of CIP was 0.25 mg/L, except for one combination at concentration ratio VAN:CIP, 0.5:0.125 mg/L. All combinations with concentrations the same as MIC_{CIP} acting alone (0.5 mg/L) displayed inhibition of bacterial growth. The same results were registered for all combinations with the concentration of VAN corresponding to the $MIC_{(VAN)}$ acting alone (1 mg/L).

The FIC index was calculated using the equation in Figure 10 to determine the kind of interaction of the VAN+CIP combination. Nine concentration ratios were included in the evaluation (see Table 7). In all categorized combinations, the indifferent effect was indicated. Nevertheless, in three combinations of VAN+CIP, with concentration ratios of VAN:CIP corresponding to 0.0625:0.5 mg/L, 1:0.03125 mg/L, and 1:0.015625 mg/L, the FIC index value was close to 1, near to additive effect.

Table 7: Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of vancomycin and ciprofloxacin. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, CIP= ciprofloxacin, VAN= vancomycin, FIC(A)= fractional inhibitory concentration of ciprofloxacin, FIC(B)= fractional inhibitory concentration of vancomycin

MIC (CIP : VAN) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
0.5 : 0.5	1.5	indifference
0.5: 0.25	1.25	indifference
0.5 : 0.125	1.125	indifference
0.5 : 0.0625	1.0625	indifference, near to additive effect
0.25 : 1	1.5	indifference
0.125 : 1	1.25	indifference
0.0625 : 1	1.125	indifference
0.03125 : 1	1.0625	indifference, near to additive effect
0.015625 : 1	1.03125	indifference, near to additive effect

5.3. Evaluation of the efficacy of vancomycin and cotrimoxazole in combination

For the preparation of VAN+COT drug combinations, solutions with different concentrations of VAN in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), solutions of COT were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. Subsequently, 10 ml per well of the bacterial inoculum was inoculated into wells.

After a 24-hour incubation period at 37°C, an evaluation of the inhibition of growth by the naked eye was done, and absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as $MIC_{(VAN)}$= 2 mg/L, and $MIC_{(COT)}$= 2 mg/L, in intra-assay evaluation. MIC of both VAN and COT within the checkerboard assay corresponded to the same value as determined in Chapter 4.1 (see Table 2). According to the EUCAST breakpoints, the tested MRSA strain is recognized as susceptible to both included antibiotics (see Table 1).

Absorbance values were used to calculate the percentage of growth inhibition using the equation in Figure 8, and acquired data were processed into a heat map. (see Figure 13).
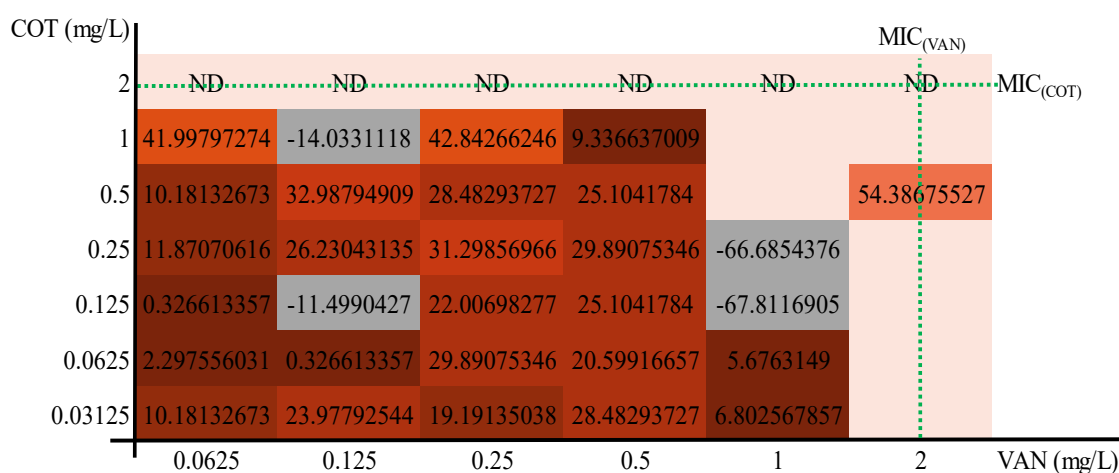
Figure 13*: Heat map of checkerboard assay of vancomycin and cotrimoxazole combination. Heat plot describing the antibacterial activity of vancomycin and cotrimoxazole in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Boxes in grey were not taken into consideration for evaluation (an error occurred during preparation). Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(VAN)}$= 1 mg/L, $MIC_{(COT)}$= 2 mg/L, COT= cotrimoxazole, VAN= vancomycin, $MIC_{(COT)}$= minimum inhibitory concentration of cotrimoxazole, $MIC_{(VAN)}$= minimum inhibitory concentration of vancomycin, ND= not determined*

It cannot be ruled out that combinations of VAN+COT, with the final concentration of COT corresponding to 2 mg/L would not lead to bacterial growth suppression. It would be sensible to repeat this assay. Nevertheless, it is apparent from Figure 13 that in none of the combinations at any concentration ratio where the final concentration of COT corresponded to 1 mg/L (½ MIC sub-inhibitory concentrations of COT), the ≥ 50% inhibition of bacterial growth has not been reached. In one well with the concentration of VAN at 1 MIC (1 mg/L) and ¼ MIC sub-inhibitory concentration of COT (0.5 mg/L), the percentage of inhibition was 58.3%, and the presence of bacterial growth was detected by visual evaluation. This tells us a possible undesirable interference between these two drugs that results in lowered activity of VAN. Overall, these results indicate this combination might not be very promising even if the assay were to be repeated with included ratios corresponding to 1 MIC of COT, and results were to reveal the additive or synergic effect.

The FIC index was calculated using the equation in Figure 10 to determine the kind of interaction of the VAN+COT combination. Seven concentration ratios where inhibition of bacterial growth was detected were included in the evaluation (see Table 8). Two combinations of VAN+COT indicated additive effect (VAN:COT, 1:0.5 mg/L, and 1:1 mg/L), and another two combinations (VAN:COT, 2:0.0625 mg/L, and 2: 0.03125 mg/L), have FIC index value close to 1, near to additive effect.

Table 8: *Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of vancomycin and cotrimoxazole. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, COT= cotrimoxazole, VAN= vancomycin, FIC(A)= fractional inhibitory concentration of cotrimoxazole, FIC(B)= fractional inhibitory concentration of vancomycin*
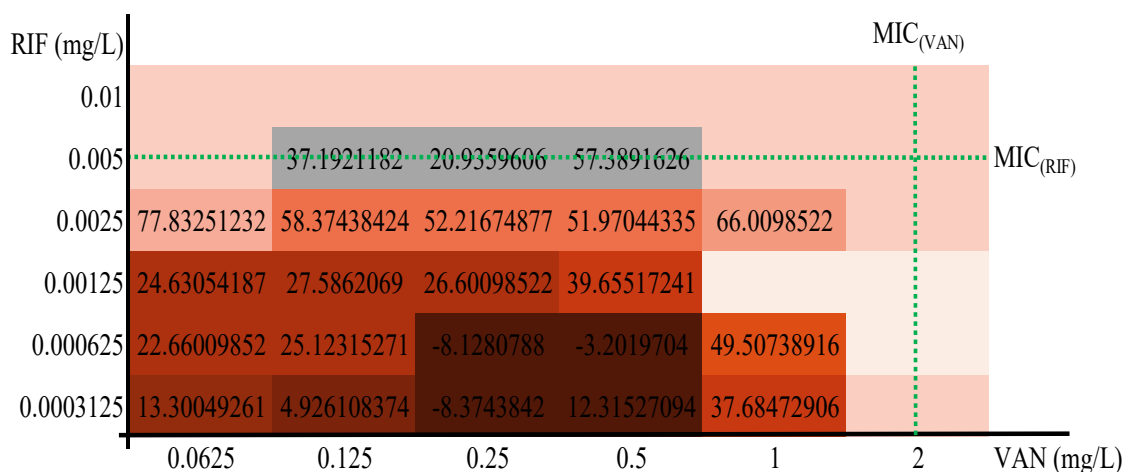
MIC (COT : VAN) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
0.03125 : 2	1.03125	indifference, near to additive effect
0.0625 : 2	1.0625	indifference, near to additive effect
0.125 : 2	1.125	indifference
0.25 : 2	1.25	indifference
0.5 : 1	1	additive
1 : 1	1	additive
1 : 2	2	indifference

5.4. Evaluation of the efficacy of vancomycin and rifampicin in combination

For the preparation of VAN+RIF drug combinations, solutions with different concentrations of VAN in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), solutions of RIF were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. Subsequently, 10 ml per well of the bacterial inoculum was inoculated into wells.

After a 24-hour incubation period at 37°C, an evaluation of the inhibition of growth by the naked eye was done, and absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as $MIC_{(VAN)}$= 2 mg/L, and $MIC_{(RIF)}$= 0.005 mg/L. MIC of both VAN and RIF within the checkerboard assay corresponded to the same value as determined in Chapter 4.1 (see Table 2). According to the breakpoints in EUCAST, MRSA strain is recognized as susceptible to both included antibiotics (see Table 1).

Absorbance values were used to calculate the percentage of growth inhibition using the equation in Figure 8, and acquired data were processed into a heat map (see Figure 14).

RIF (mg/L)	0.0625	0.125	0.25	0.5	1	2	VAN (mg/L)
0.01							
0.005		37.1921182	20.9359606	57.3891626			$MIC_{(RIF)}$
0.0025	77.83251232	58.37438424	52.21674877	51.97044335	66.0098522		
0.00125	24.63054187	27.5862069	26.60098522	39.65517241			
0.000625	22.66009852	25.12315271	-8.1280788	-3.2019704	49.50738916		
0.0003125	13.30049261	4.926108374	-8.3743842	12.31527094	37.68472906		

Figure 14: Heat map of checkerboard assay of vancomycin and rifampicin combination. Heat plot describing the antibacterial activity of vancomycin and rifampicin in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Boxes in grey were not taken into consideration for evaluation (an error occurred during preparation). Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(VAN)}$= 1 mg/L, $MIC_{(RIF)}$= 0.005 mg/L, RIF= rifampicin, VAN= vancomycin, $MIC_{(RIF)}$= minimum inhibitory concentration of rifampicin, $MIC_{(VAN)}$= minimum inhibitory concentration of vancomycin.

Three combinations of VAN+RIF have been excluded from evaluation and data interpretation (VAN:RIF, 0.125:0.005 mg/L, 0.25:0.005 mg/L, and 0.5:0.005 mg/L). In corresponding wells, no bacterial growth was evident after visual inspection. Some sort of error occurred during spectrophotometric measurement. From Figure 14 can be seen that in all other combinations with final concentrations of VAN and RIF corresponding to MIC of VAN and RIF acting alone, the bacterial growth was completely inhibited. In other combinations, the percentage of bacterial growth inhibition in combinations with sub-inhibitory concentrations of both VAN and RIF was mostly under 50%. In all combinations of VAN+RIF with ½ MIC sub-inhibitory concentrations of RIF (0.0025 mg/L), the $\geq$ 50% inhibition of bacterial growth was achieved. In two combinations with ½ MIC sub-inhibitory concentration of VAN (1 mg/L) and sub-inhibitory concentrations of RIF (¼ and ⅛ MIC, i.e. 0.0025 mg/L and 0.00125 mg/L), the > 60% and > 90% inhibition of bacterial growth was achieved, respectively.

In some VAN+RIF combinations, concentration ratios corresponding to ⅛ and $\frac{1}{16}$ MIC sub-inhibitory concentrations of RIF and ¼ and ⅛ MIC sub-inhibitory concentrations of VAN, the bacterial growth was potentiated (negative values for % of bacterial growth inhibition). Sub-inhibitory concentration of antibiotic drugs with bactericidal effect leads to bacterial stress response, and enhancement of bacterial metabolic activity. In addition, bacterial metabolism and respiration are interconnected with bacterial growth rate. (76; 77) As such, it is possible that instead of inhibition of bacterial growth, an increase in bacterial growth can occur after the exposition of bacteria to sub-inhibitory concentrations of antibiotic drugs, which probably happened in the case of this combination. In Figure 14, negative values of the % of inhibition describe this phenomenon.

The FIC index was calculated using the equation in Figure 10 to determine the kind of interaction of the VAN+RIF combination. Seven concentration ratios, where inhibition of bacterial growth was detected, were included in the evaluation (see Table 9). Six categorized combinations indicated an indifferent effect and one additive effect at VAN:RIF concentration ratio 1:0.00125 mg/L. Out of six combinations that showed indifference, three combinations (VAN:RIF, 2:0.0003125 mg/L, 0.125:0.005 mg/L, and 0.0625:0.005 mg/L) have FIC index values close to 1, near to additive effect.

Table 9: *Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of vancomycin and rifampicin. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, RIF= rifampicin, VAN= vancomycin, FIC(A)= fractional inhibitory concentration of vancomycin, FIC(B)= fractional inhibitory concentration of rifampicin*

MIC (VAN : RIF) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
2 : 0.0025	1.5	indifference
2 : 0.000625	1.125	indifference
2 : 0.0003125	1.0625	indifference, near to additive effect
1 : 0.005	1.5	indifference
1 : 0.00125	0.75	additive
0.0625 : 0.005	1.03125	indifference, near to additive effect

5.5. Evaluation of the efficacy of linezolid and ciprofloxacin in combination

For the preparation of LIN+CIP drug combinations, solutions with different concentrations of LIN in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), solutions of CIP were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. Subsequently, 10 ml per well of the bacterial inoculum was inoculated into wells.

After a 24-hour incubation period at 37°C, an evaluation of the inhibition of growth by the naked eye was done, and absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as $MIC_{(LIN)}$= 4 mg/L and $MIC_{(CIP)}$= 0.5 mg/L. $MIC_{(CIP)}$ within the checkerboard assay corresponded to the same value as determined in Chapter 4.1, $MIC_{(LIN)}$ dropped from 8 mg/L to 4 mg/L (see Table 2). According to the EUCAST breakpoints, MRSA strain is recognized as susceptible to both included antibiotics (see Table 1).

Absorbance values were used to calculate the percentage of growth inhibition using the equation in Figure 8, and acquired data were processed into a heat map (see Figure 15).
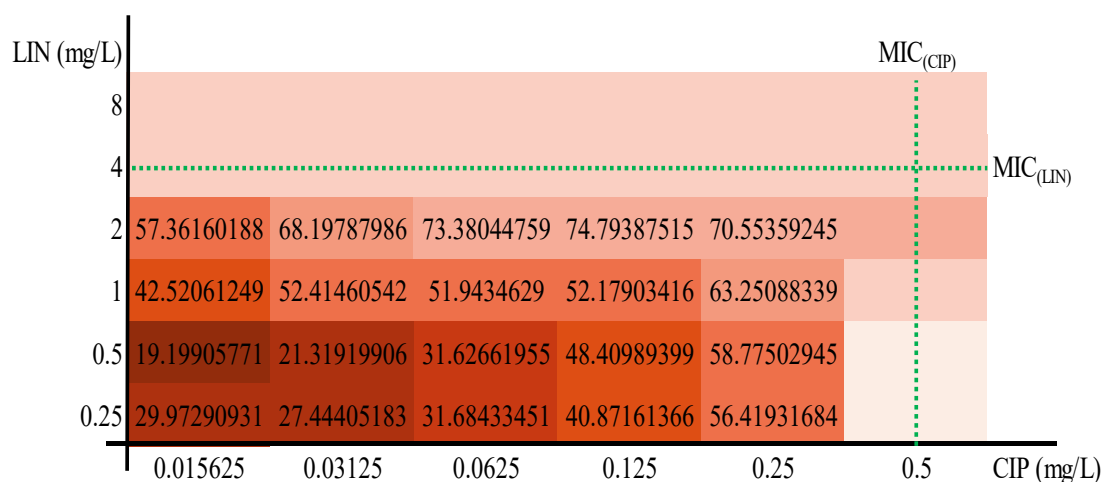
Figure 15: *Heat map of checkerboard assay of linezolid and ciprofloxacin combination. Heat plot describing the antibacterial activity of linezolid and ciprofloxacin in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(LIN)}$= 4 mg/L, $MIC_{(CIP)}$= 0.5 mg/L, LIN= linezolid, CIP= ciprofloxacin, $MIC_{(LIN)}$= minimum inhibitory concentration of linezolid, $MIC_{(CIP)}$= minimum inhibitory concentration of ciprofloxacin*

One combination (LIN:CIP, 2:0.5 mg/L) was excluded from the evaluation. In corresponding well, no bacterial growth was evident after visual inspection. Some sort of error occurred during spectrophotometric detection. In all other combinations corresponding to MIC of LIN and CIP acting alone, bacterial growth was not detected. In combinations of LIN+CIP with ½ MIC sub-inhibitory concentrations of both LIN (2 mg/L) and CIP (0.25 mg/L), $\geq 50\%$ inhibition of bacterial growth was registered.

The FIC index was calculated using the equation in Figure 10 to determine the kind of interaction of the LIN+CIP combination. Eight concentration ratios where inhibition of bacterial growth was detected were included in the evaluation (see Table 10). All categorized combinations indicated indifferent effect. Similarly to the VAN+RIF combination, out of eight tested combinations, three combinations (LIN:CIP, 4:0.015625 mg/L, 4:0.03125 mg/L, and 0.25:0.5 mg/L) have FIC index values close to 1, near to additive effect.

Table 10: *Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of linezolid and ciprofloxacin. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, LIN= linezolid, CIP= ciprofloxacin, FIC(A)= fractional inhibitory concentration of ciprofloxacin, FIC(B)= fractional inhibitory concentration of linezolid*
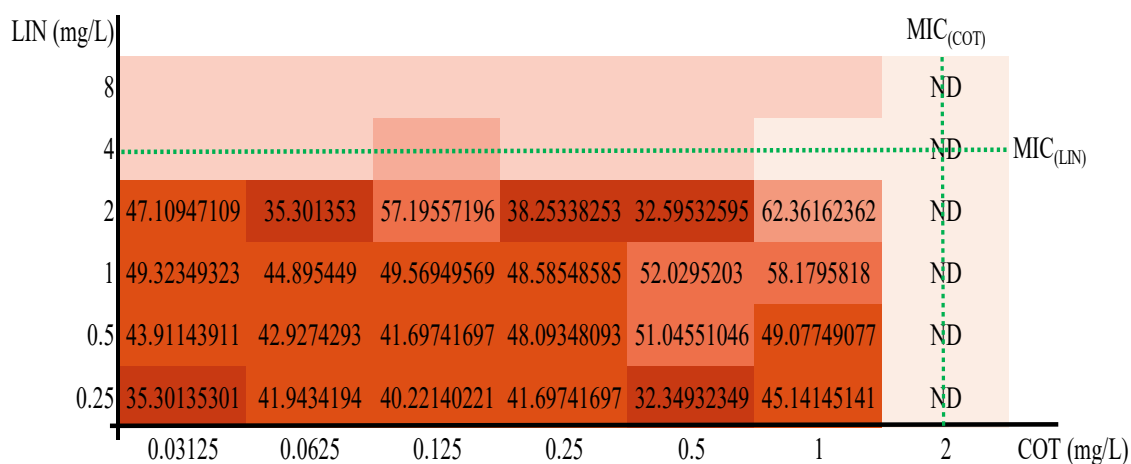
MIC (CIP : LIN) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
0.0156 : 4	1.0312	indifference, near to additive effect
0.03125 : 4	1.0625	indifference, near to additive effect
0.0625 : 4	1.125	indifference
0.125 : 4	1.25	indifference
0.25 : 4	1.5	indifference
0.5 : 1	1.25	indifference
0.5 : 0.5	1.125	indifference
0.5 : 0.25	1.0625	indifference, near to additive effect

5.6. Evaluation of the efficacy of linezolid and cotrimoxazole in combination

For the preparation of LIN+COT drug combinations, solutions with different concentrations of COT in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), solutions of LIN were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. Subsequently, 10 ml per well of the bacterial inoculum was inoculated into wells.

After 24 hours of incubation at 37°C, an evaluation of the inhibition of growth by the naked eye was done, and absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as $MIC_{(LIN)}$= 4 mg/L, and $MIC_{(COT)}$= 2 mg/L in intra-assay evaluation. Similarly to the VAN+COT combination (see Chapter 5.3), combinations of LIN+COT with the final concentration of COT corresponding to 2 mg/L were not included in the assay. $MIC_{(COT)}$ within the checkerboard assay corresponded to the same value as determined in Chapter 4.1, while $MIC_{(LIN)}$ jumped from 8 mg/L to 4 mg/L (see Table 2). According to the breakpoints in EUCAST, MRSA strain is recognized as susceptible to both included antibiotics (see Table 1).

Absorbance values were used to calculate the percentage of growth inhibition using the equation in Figure 8, and acquired data were processed into a heat map (see Figure 16).

LIN (mg/L)	0.03125	0.0625	0.125	0.25	0.5	1	2 $MIC_{(COT)}$
8							ND
4							ND — $MIC_{(LIN)}$
2	47.10947109	35.301353	57.19557196	38.25338253	32.59532595	62.36162362	ND
1	49.32349323	44.895449	49.56949569	48.58548585	52.0295203	58.1795818	ND
0.5	43.91143911	42.9274293	41.69741697	48.09348093	51.04551046	49.07749077	ND
0.25	35.30135301	41.9434194	40.22140221	41.69741697	32.34932349	45.14145141	ND

COT (mg/L)

Figure 16*: Heat map of checkerboard assay of linezolid and cotrimoxazole combination. Heat plot describing the antibacterial activity of linezolid and cotrimoxazole in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(LIN)}$= 4 mg/L, $MIC_{(COT)}$= 2 mg/L, LIN= linezolid, COT= cotrimoxazole, $MIC_{(LIN)}$= minimum inhibitory concentration of linezolid, $MIC_{(COT)}$= minimum inhibitory concentration of cotrimoxazole, ND= not determined*

Evaluation of this combination is similar to the VAN+COT combination— it cannot be determined whether the inhibition of bacterial growth occurred in combinations with the concentration of COT= 2 mg/L. It is apparent that only in some combinations corresponding to ½ MIC sub-inhibitory concentrations of LIN, the ≥ 50% inhibition of bacterial growth was registered. In two combinations with ½ MIC sub-inhibitory concentrations of COT from the total four combinations, a ≥ 50% inhibition of bacterial growth was registered.

The FIC index was calculated using the equation in Figure 10 to determine the kind of interaction of the LIN+COT combination. The evaluation included six concentration ratios where inhibition of bacterial growth was detected (see Table 11). Three categorized combinations (LIN:COT, 4:0.0625 mg/L, 4: 0.03125 mg/L, and 4:0.125 mg/L, have FIC index value close to 1 (near to additive effect), and three combinations indicate indifference.

Table 11: Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of linezolid and cotrimoxazole. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, LIN= linezolid, COT= cotrimoxazole, FIC(A)= fractional inhibitory concentration of cotrimoxazole, FIC(B)= fractional inhibitory concentration of linezolid

MIC (COT : LIN) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
1 : 4	1.5	indifference
0.5 : 4	1.25	indifference
0.25 : 4	1.125	indifference
0.125 : 4	1.0625	indifference, near to additive effect
0.0625 : 4	1.03125	indifference, near to additive effect
0.03125 : 4	1.015625	indifference, near to additive effect

5.7. Evaluation of the efficacy of linezolid and rifampicin in combination

For the preparation of LIN+RIF drug combinations, solutions with different concentrations of RIF in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), solutions of LIN were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. Subsequently, 10 ml per well of the bacterial inoculum was inoculated into wells.

After a 24-hour incubation period at 37°C, an evaluation of the inhibition of growth by the naked eye was done, and absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as MIC$_{(LIN)}$= 4 mg/L, and MIC$_{(RIF)}$= 0.00 5mg/L. MIC$_{(RIF)}$ within the checkerboard assay corresponded to the same value as determined in Chapter 4.1, while MIC$_{(LIN)}$ dropped from 8 mg/L to 4 mg/L (see Table 2). According to the EUCAST breakpoints, MRSA strain is recognized as susceptible to both included antibiotics (see Table 1).

Absorbance values were used to calculate the percentage of growth inhibition using the equation in Figure 8, and acquired data were processed into a heat map (see Figure 17).
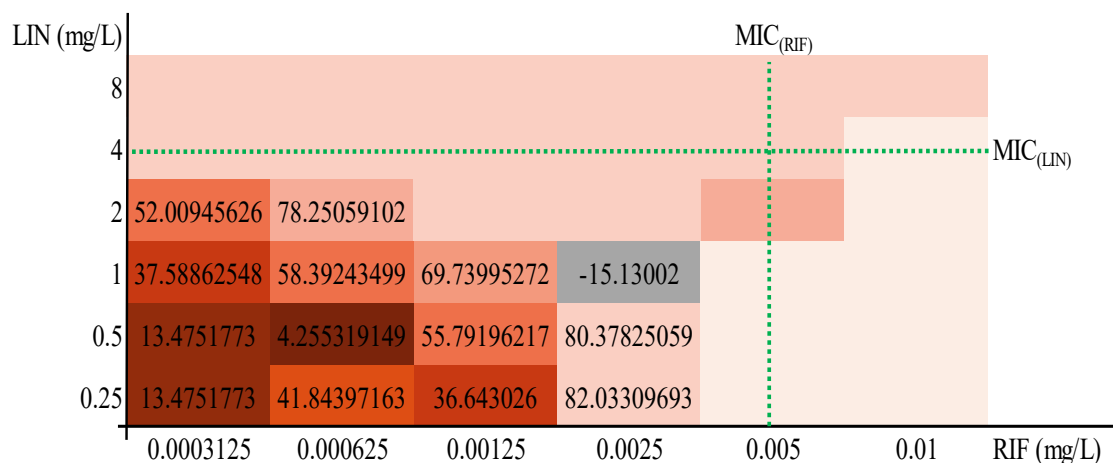
Figure 17*: Heat map of checkerboard assay of linezolid and rifampicin combination. Heat plot describing the antibacterial activity of linezolid and rifampicin in combination against* Staphylococcus aureus, *ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. The box in grey were not taken into consideration for evaluation (an error occurred during preparation). Green dotted lines describe the MIC of antibiotics. Legend: MIC$_{(LIN)}$= 4 mg/L, MIC$_{(RIF)}$= 0.005 mg/L, LIN= linezolid, RIF= rifampicin, MIC$_{(LIN)}$= minimum inhibitory concentration of linezolid, MIC$_{(RIF)}$= minimum inhibitory concentration of rifampicin*

In the combination LIN+RIF corresponding to ratio LIN:RIF, 2:0.005 mg/L, bacterial growth was not registered after evaluation by the naked eye. Nevertheless, after data processing, the inhibition of the growth corresponded only to 76.832%. In combination at a concentration ratio LIN:RIF, 1:0.0025 mg/L, a potentiation of bacterial growth was registered. It is most likely an error occurred during preparation, however, there might be a possibility of a negative mutual interaction. Similarly to the VAN+RIF combination, in all other combinations of LIN+RIF with the final concentrations of LIN and RIF corresponding to MIC of LIN and RIF acting alone, the total inhibition of bacterial growth (analysis of data from spectrophotometric detection) was registered.

In the combination LIN+RIF corresponding to ratio LIN:RIF, 1:0.0025 mg/L, bacterial growth was registered. After data processing, it was evident that this combination potentiate bacterial growth. In all other combinations of LIN+RIF with ½ MIC sub-inhibitory concentrations of both LIN (2 mg/L) and RIF (0.25 mg/L), the ≥ 50% inhibition of the bacterial growth was registered.

The FIC index was calculated using the equation in Figure 10 to determine the kind of interaction of the LIN+RIF combination. Seven concentration ratios where inhibition of bacterial growth was detected were included in the evaluation (see Table 12). Three categorized combinations of LIN+RIF, with concentration ratio LIN:RIF corresponding to 2:0.00125 mg/L, 2:0.0025 mg/L, and 0.5:0.005 mg/L, indicate additive effect. One categorized combination with LIN:RIF concentration ratio 4:0.003125 mg/L has an FIC value close to 1, (near to additive effect), and the remaining two categorized combinations indicate indifference.

Table 12: *Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of linezolid and rifampicin. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, LIN= linezolid, RIF= rifampicin, FIC(A)= fractional inhibitory concentration of rifampicin, FIC(B)= fractional inhibitory concentration of linezolid*

| MIC (RIF : LIN) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
0.0003125 : 4	1.0625	indifference, near to additive effect
0.000625 : 4	1.125	indifference
0.00125 : 2	0.75	additive
0.0025 : 2	1.00	additive
0.005 : 1	1.25	indifference
0.005 : 0.5	1	additive
0.005 : 0.25	1.125	indifference

5.8. Evaluation of the efficacy of ciprofloxacin and cotrimoxazole in combination

For the preparation of CIP+COT drug combinations, solutions with different concentrations of COT in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), solutions of CIP were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. Subsequently, 10 ml per well of the bacterial inoculum was inoculated into wells.

After 24 hours of incubation at 37°C, evaluation of the inhibition of growth by the naked eye was done, and absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as $MIC_{(CIP)}$= 0.5 mg/L, and $MIC_{(COT)}$= 2 mg/L, in intra-assay evaluation. Similarly to the VAN+COT (see Chapter 5.3) and LIN+COT (see Chapter 5.6) combinations, combinations of CIP+COT with the final concentration of COT corresponding to 2 mg/L were not included in the assay. MIC of both CIP and COT within the checkerboard assay corresponded to the same value as determined in Chapter 4.1 (see Table 2). According to the EUCAST breakpoints, MRSA strain is recognized as susceptible to both included antibiotics (see Table 1).

Absorbance values were used to calculate the percentage of growth inhibition using the equation in Figure 8, and acquired data were processed into a heat map (see Figure 18).
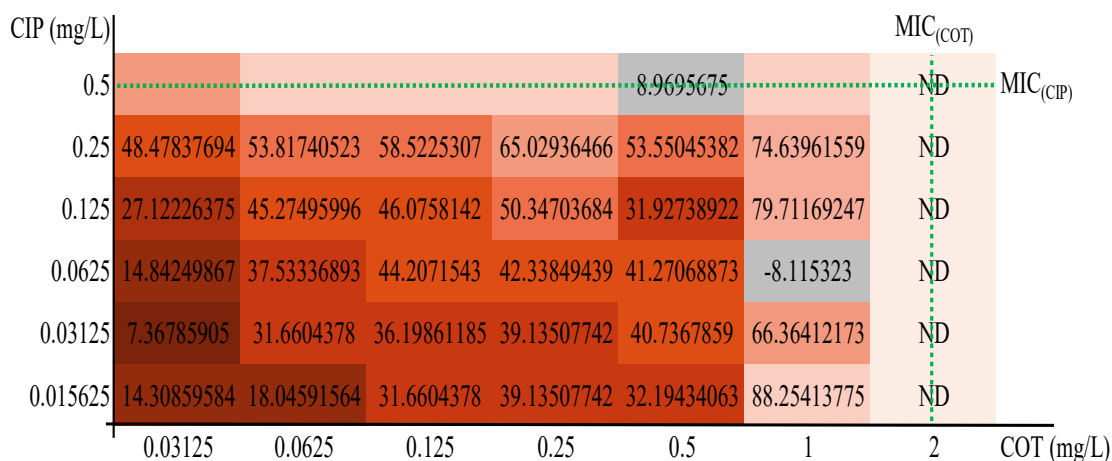
Figure 18: *Heat map of checkerboard assay of ciprofloxacin and cotrimoxazole combination. Heat plot describing the antibacterial activity of ciprofloxacin and cotrimoxazole in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Boxes in grey were not taken into consideration for evaluation (an error occurred during preparation). Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(CIP)}$= 0.5 mg/L, $MIC_{(COT)}$= 2 mg/L, CIP= ciprofloxacin, COT= cotrimoxazole, $MIC_{(CIP)}$= minimum inhibitory concentration of ciprofloxacin, $MIC_{(COT)}$= minimum inhibitory concentration of cotrimoxazole, ND= not determined*

Evaluation of this combination is similar to VAN+COT and LIN+COT combinations— the inhibition of bacterial growth at a concentration of COT= 2 mg/L cannot be determined.

It is evident from Figure 18 that the % of inhibition above 50% was detected only for ½ MIC sub-inhibitory concentrations of CIP. In one combination corresponding to the concentration ratio CIP:COT, 0.0625:1 mg/L, the potentiation of bacterial growth was registered, and another combination at concentration ratio CIP:COT, 0.5:0.5 mg/L registered only 8.97% inhibition. Although an error probably occurred during preparation, a possible negative mutual interaction cannot be completely ruled out. While all combinations with the concentration of CIP corresponding to 1 MIC registered total inhibition of bacterial growth, it is important to point out the combination at concentration ratio CIP:COT, 0.5:0.03125 mg/L, in which the % of inhibition, determined by spectrophotometric measurement, was 69%, which is fairly lower to the rest of the combinations (average % of inhibition was 87%)

The FIC index was calculated using the equation in Figure 10 to determine the kind of interaction of the CIP+COT combination. Five concentration ratios where inhibition of bacterial growth was detected were included in the evaluation (see Table 13). Three categorized combinations at CIP:COT concentrations ratio of 0.5:0.125 mg/L, 0.5: 0.0625 mg/L, and 0.5:0.03125 mg/L, have FIC index value close to 1, near to additive effect, while the remaining combinations registered indifference.

Table 13: *Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction ciprofloxacin and cotrimoxazole. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, CIP= ciprofloxacin, COT= cotrimoxazole, FIC(A)= fractional inhibitory concentration of ciprofloxacin, FIC(B)= fractional inhibitory concentration of cotrimoxazole*

MIC (CIP : COT) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
0.5 : 1	1.5	indifference
0.5 : 0.25	1.125	indifference
0.5 : 0.125	1.0625	indifference, near to additive effect
0.5 : 0.0625	1.03125	indifference, near to additive effect
0.5 : 0.03125	1.015625	indifference, near to additive effect

5.9. Evaluation of the efficacy of ciprofloxacin and rifampicin in combination

For the preparation of CIP+RIF drug combinations, solutions with different concentrations of RIF in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), solutions of CIP were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. Subsequently, 10 ml per well of the bacterial inoculum was inoculated into wells.

After a 24-hour incubation period at 37°C, an evaluation of the inhibition of growth by the naked eye was done, and absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as $MIC_{(CIP)}$= 0.25 mg/L, and $MIC_{(RIF)}$= 0.01 mg/L. $MIC_{(CIP)}$ within the checkerboard assay corresponded to the same value as determined in Chapter 4.1, and $MIC_{(RIF)}$ jumped from 0.005 mg/L to 0.01 mg/L (see Table 2). According to the EUCAST breakpoints, MRSA strain is recognized as susceptible to both included antibiotics (see Table 1).

Absorbance values were used to calculate the percentage of growth inhibition using the equation in Figure 8, and acquired data were processed into a heat map. (see Figure 19).
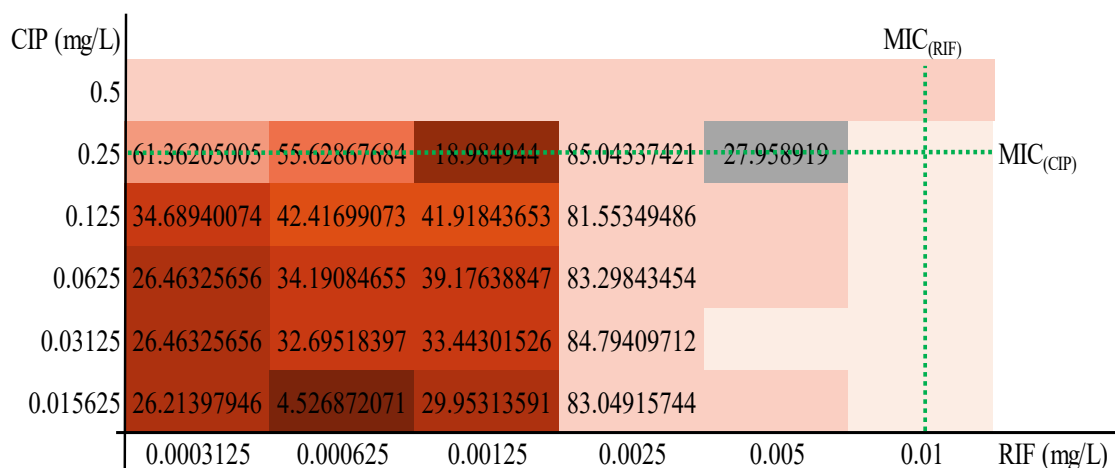
Figure 19: *Heat map of checkerboard assay of ciprofloxacin and rifampicin combination. Heat plot describing the antibacterial activity of ciprofloxacin and rifampicin in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. The box in grey was not taken into consideration for evaluation (an error occurred during preparation). Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(CIP)}$= 0.5 mg/L, $MIC_{(RIF)}$= 0.01 mg/L, CIP= ciprofloxacin, RIF= rifampicin, $MIC_{(CIP)}$= minimum inhibitory concentration of ciprofloxacin, $MIC_{(RIF)}$= minimum inhibitory concentration of rifampicin*

Heat map of CIP+RIF combination shows that for some combinations of CIP+RIF with concentration of CIP corresponding to $MIC_{(CIP)}$ acting alone (0.25 mg/L), the inhibition of bacterial growth was not registered. The inhibition of the growth in combinations where CIP concentration corresponded to ½ subMIC of CIP acting alone, the percentage of growth inhibition ranged from 34.69% to over 90%. This indicates the loss of efficacy of CIP in combination with RIF. However, this was not the case for combinations of CIP+RIF with the concentration of RIF corresponding to $MIC_{(RIF)}$ acting alone (0.01 mg/L). In combinations of CIP+RIF with concentrations of RIF corresponding to ½ MIC sub-inhibitory concentrations of RIF (0.005 mg/L), the bacterial growth was also not registered by the naked eye, and additionally, in CIP+RIF combinations with ¼ MIC sub-inhibitory concentrations of RIF (0.0025 mg/L) was registered inhibition of bacterial growth over 50%. This means that while CIP is antagonized by RIF, RIF is potentiated by CIP. Two combinations at concentration ratio CIP:RIF, 0.25:0.005 mg/L and 0.25:0.00125 mg/L registered lower % of inhibition (27.96% and 18.98% respectively). While it appears that an error during preparation occurred in the case of the first combination, the same cannot be said with certainty about the second combination.

The FIC index was calculated using the equation in Figure 10 to determine the kind of interaction of the CIP+RIF combination. Four concentration ratios where inhibition of bacterial growth was detected were included in the evaluation (see Table 14). All categorized combinations (CIP:RIF, 0.005:0.125 mg/L, 0.005:0.0625 mg/L, 0.005:0.03125 mg/L, and 0.005:0.0015625 mg/L) indicate additive effect, confirming the potentiation of RIF by CIP.

Table 14: Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction ciprofloxacin and rifampicin. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, RIF= rifampicin, CIP= ciprofloxacin, FIC(A)= fractional inhibitory concentration of rifampicin, FIC(B)= fractional inhibitory concentration of ciprofloxacin.

MIC (RIF : CIP) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
0.005 : 0.125	1	additive
0.005 : 0.0625	0.75	additive
0.005 : 0.03125	0.625	additive
0.005 : 0.015625	0.5625	additive

5.10. Evaluation of the efficacy of cotrimoxazole and rifampicin in combination

For the preparation of COT+RIF drug combinations, solutions with different concentrations of COT in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), solutions of RIF were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. Subsequently, 10 ml per well of the bacterial inoculum was inoculated into wells.

After 24 hours of incubation at 37°C, the inhibition of growth by the naked eye was evaluated, and absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as $MIC_{(COT)}= 2$ mg/L, and $MIC_{(RIF)}= 0.01$ mg/L. Similarly to the VAN+COT (see Chapter 5.3), LIN+COT (see Chapter 5.6), and CIP+COT (see Chapter 5.8) combinations, combinations of COT+RIF with the final concentration of COT corresponding to 2 mg/L were not included in the assay. The $MIC_{(COT)}$ within the checkerboard assay corresponded to the same value as determined in Chapter 4.1, while the $MIC_{(RIF)}$ jumped from 0.005 mg/L to 0.01 mg/L (see Table 2). According to the EUCAST breakpoints, MRSA strain is recognized as susceptible to both included antibiotics (see Table 1).

Absorbance values were used to calculate the percentage of growth inhibition using the equation in Figure 8, and acquired data were processed into a heat map (see Figure 20).
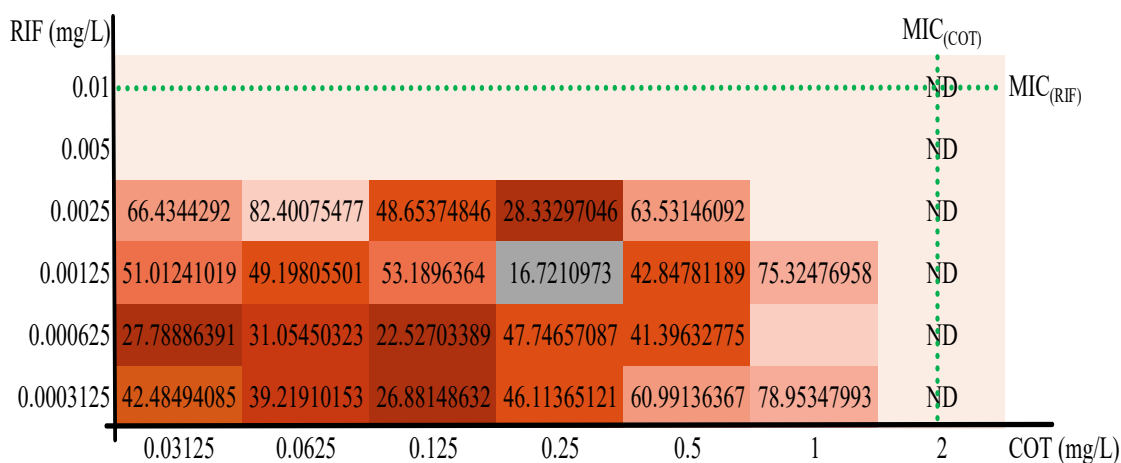
***Figure 20**: Heat map of checkerboard assay of cotrimoxazole and rifampicin combination. Heat plot describing the antibacterial activity of cotrimoxazole and rifampicin in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(COT)}= 2$ mg/L, $MIC_{(RIF)}= 0.01$ mg/L, COT= cotrimoxazol, RIF= rifampicin, $MIC_{(COT)}=$ minimum inhibitory concentration of cotrimoxazol, $MIC_{(RIF)}=$ minimum inhibitory concentration of rifampicin., ND= not determined*

The efficacy of the antibacterial action of COT+RIF combinations with the final concentration of COT= 2 mg/L cannot be determined. However, Figure 20 demonstrates that for all combinations of COT+RIF with the final concentration of RIF corresponding to MIC$_{(RIF)}$ acting alone (0.01 mg/L), the total inhibition of bacterial growth by the naked eye and by spectrophotometric detection was registered. Subsequently, in combinations of COT+RIF with ½ MIC sub-inhibitory concentrations of RIF (0.005 mg/L), the total inhibition of bacterial growth was registered, and in all combinations of COT+RIF with ½ MIC sub-inhibitory concentrations of COT (1 mg/L) was registered partial inhibition of bacterial growth over 50% compared to positive control.

The FIC index was calculated using the equation in Figure 10 to determine the kind of interaction of the COT+RIF combination. Seven concentration ratios, where inhibition of bacterial growth was detected, were included in the evaluation (see Table 15). Six categorized combinations (COT:RIF, 1:0.005 mg/L, 1:0.0025 mg/L, 0.5:0.005 mg/L, 0.25:0.005 mg/L, 0.125:0.005 mg/L, and 0.0625:0.005 mg/L) indicate additive effect. The last categorized combination with a concentration ratio of COT:RIF, 0.03125:0.005 mg/L has an FIC index value close to 0.5, near to synergy.

Table 15: *Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of cotrimoxazole and rifampicin. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, RIF= rifampicin, COT= cotrimoxazole, FIC(A)= fractional inhibitory concentration of cotrimoxazole, FIC(B)= fractional inhibitory concentration of rifampicin.*

MIC (COT : RIF) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
1 : 0.0025	0.75	additive
1 : 0.000625	0.5625	additive
0.5 : 0.005	0.75	additive
0.25 : 0.005	0.625	additive
0.125 : 0.005	0.5625	additive
0.0625 : 0.005	0.53125	additive
0.03125 : 0.005	0.515625	additive, near to synergy

5.11. Evaluation of the efficacy of daptomycin and vancomycin, daptomycin and linezolid, daptomycin and ciprofloxacin, and daptomycin and rifampicin, in combination

For the preparation of DAP+VAN drug combinations, solutions with different concentrations of VAN in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), and solutions of DAP were pipetted in a horizontal direction (six rows). For the preparation of DAP+LIN, DAP+CIP, and DAP+RIF drug combinations, solutions with different concentrations of DAP in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), solutions of LIN, CIP, and RIF were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. 10 ml of the bacterial inoculum per well was inoculated into wells.

After a 24-hour incubation period at 37°C, an evaluation of the inhibition of growth by the naked eye was done, and absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as $MIC_{(VAN)}$= 1 mg/L, $MIC_{(LIN)}$= 2 mg/L, $MIC_{(CIP)}$= 0.25 mg/L, $MIC_{(RIF)}$= 0.005 mg/L. However, in all checkerboard assays with combinations including DAP, the $MIC_{(DAP)}$ corresponded to > 8 mg/L. This value of MIC does not match with previously determined values (see Table 2)— the loss of activity of DAP can be speculated. Nevertheless, within the checkerboard assays, the internal evaluation of DAP acting alone, and DAP acting in combination has been doneThe attention has been paid to the shift of the final concentration of the drug in combination compared to the MIC of the drug acting alone. Therefore, registration of MIC not fully corresponding to the predicted value should not be considered a drawback.

MIC of VAN, CIP, and RIF within the checkerboard assay corresponded to the same value as determined in Chapter 4.1. However, the MIC of LIN decreased significantly from 8 mg/L to 2 mg/L (see Table 2). According to the EUCAST breakpoints, the MRSA strain is recognized as susceptible to all the above-mentioned antibiotics except DAP (see Table 1). To ascertain whether a mistake occurred in the process of preparation of daptomycin stock solution and dilutions for combination DAP+VAN and DAP+CIP, a fresh stock solution of DAP and dilutions were prepared for DAP+LIN and DAP+RIF combinations. However, the results were the same as in previous assays— $MIC_{(DAP)}$> 8 mg/L. As such, the use of daptomycin for further testing was abandoned, and evaluation of the remaining combinations of DAP+TIG, and DAP+COT was not carried out.

As the $MIC_{(DAP)}$ could not be properly determined, the FIC index of these antibiotic combinations also could not be calculated. However, were the $MIC_{(DAP)}$ assumed to be 16 mg/L, the FIC index values could be calculated using the equation in Figure 10, and provide at least an approximate evaluation of the efficacy of these combinations, as described in Table 16, Table 17, Table 18, and Table 19. Heat maps of each combination (Figure 21, Figure 22, Figure 23, and Figure 24) were created to assess the efficacy of combinations with sub-inhibitory concentrations of DAP.

The heat map of the DAP+VAN combination (Figure 21) demonstrates that combinations with a sub-inhibitory concentration of both antibiotics display that in most DAP+VAN combinations with sub-inhibitory MIC of DAP and VAN, the potentiation of the bacterial growth was registered. This outcome is similar to the VAN+RIF combination (see Chapter 5.4). Negative values of the percentage of inhibition represent that the density of bacterial growth in wells with combinations of antibiotics was greater than that of positive control. In addition, as shown in Figure 20, except for the concentration ratio corresponding to DAP:VAN, 4:1 mg/L, the activity of VAN with a final concentration of 1 mg/L in all other combinations with RIF was reduced.
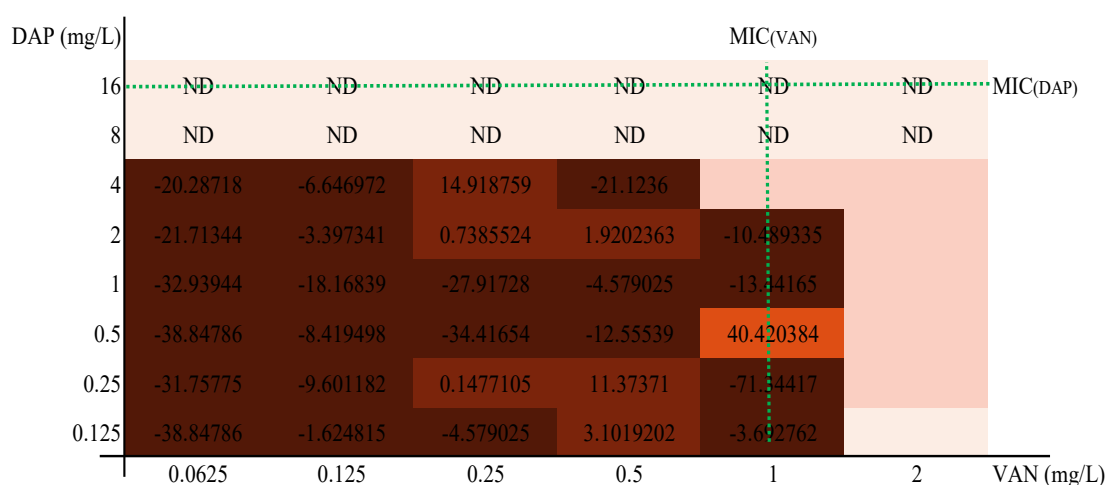
Figure 21*: Heat map of checkerboard assay of daptomycin and vancomycin combination. Heat plot describing the antibacterial activity of daptomycin and vancomycin in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(DAP)}$= 16 mg/L, $MIC_{(VAN)}$= 1 mg/L, DAP= daptomycin, VAN= vancomycin, $MIC_{(DAP)}$= minimum inhibitory concentration of daptomycin, $MIC_{(VAN)}$= minimum inhibitory concentration of vancomycin, ND= not determined*

Table 16*: Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of daptomycin and vancomycin. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, DAP= daptomycin, VAN= vancomycin, FIC(A)= final inhibitory concentration of daptomycin, FIC(B)= fractional inhibitory concentration of vancomycin*

MIC (DAP: VAN) mg/L	$\sum$FIC = FIC (A) + FIC (B)	Interpretation
4 : 1	1.25	indifference

In Figure 22 can be seen that DAP does not increase the efficacy of LIN. In combinations with the concentration of LIN corresponding to ½ MIC sub-inhibitory concentration, the percentage of inhibition values barely exceeded 50%.
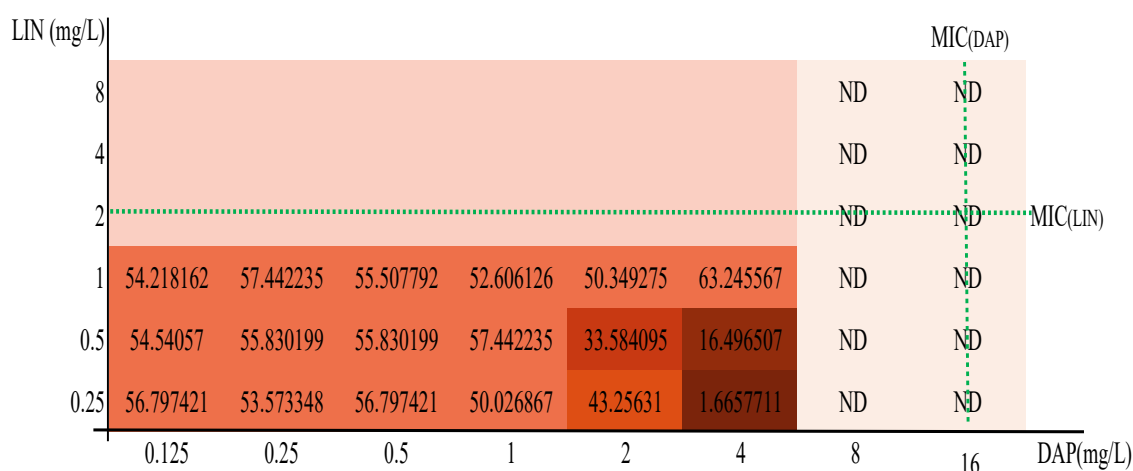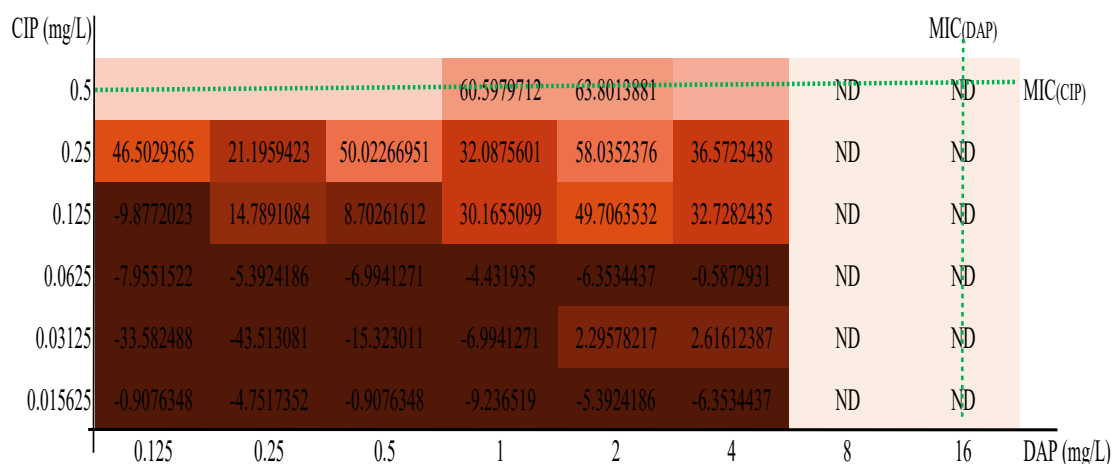

Figure 22: *Heat map of checkerboard assay of daptomycin and linezolid combination. Heat plot describing the antibacterial activity of daptomycin and linezolid in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(DAP)}$ =16 mg/L, $MIC_{(LIN)}$ =2 mg/L, DAP= daptomycin, LIN= linezolid, $MIC_{(DAP)}$= minimum inhibitory concentration of daptomycin, $MIC_{(LIN)}$= minimum inhibitory concentration of linezolid, ND= not determined*

In Table 17, the FIC index values of four out of six DAP+LIN combinations with the concentration of LIN corresponding to $MIC_{(LIN)}$ (2 mg/L), the evaluation of interaction was that of near additive effect.

Table 17: *Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of daptomycin and linezolid. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, DAP= daptomycin, LIN= linezolid FIC(A)= final inhibitory concentration of daptomycin, FIC(B)= fractional inhibitory concentration of linezolid*

MIC (DAP : LIN) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
0.125 : 2.0	1.0078125	indifference, near to additive effect
0.25 : 2.0	1.015625	indifference, near to additive effect
0.5 : 2.0	1.03125	indifference, near to additive effect
1.0 : 2.0	1.0625	indifference, near to additive effect
2.0 : 2.0	1.125	indifference
4.0 : 2.0	1.25	indifference

From Figure 23 can be seen that for the DAP+CIP combination at concentration ratios 1:0.5 mg/L and 2:0.5 mg/L, in which the final concentration of CIP corresponds to $MIC_{(CIP)}$, the presence of bacterial growth was registered. Overall, the % of inhibition at sub-inhibitory concentrations of both DAP and CIP was very low, even going into negative values. Thus, it can be concluded that these combinations are unfavorable. The activity of CIP was not potentiated by the combination with DAP (no total inhibition of the bacterial growth was registered in combinations with the final concentration of CIP corresponding to sub-MIC of CIP).

CIP (mg/L)	0.125	0.25	0.5	1	2	4	8	16	DAP (mg/L)
0.5				60.5979712	63.8013881		ND	ND	$MIC_{(CIP)}$
0.25	46.5029365	21.1959423	50.02266951	32.0875601	58.0352376	36.5723438	ND	ND	
0.125	-9.8772023	14.7891084	8.70261612	30.1655099	49.7063532	32.7282435	ND	ND	
0.0625	-7.9551522	-5.3924186	-6.9941271	-4.431935	-6.3534437	-0.5872931	ND	ND	
0.03125	-33.582488	-43.513081	-15.323011	-6.9941271	2.29578217	2.61612387	ND	ND	
0.015625	-0.9076348	-4.7517352	-0.9076348	-9.236519	-5.3924186	-6.3534437	ND	ND	

Figure 23: *Heat map of checkerboard assay of daptomycin and ciprofloxacin combination. Heat plot describing the antibacterial activity of daptomycin and ciprofloxacin in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(DAP)}$= 16 mg/L, $MIC_{(CIP)}$= 0.5 mg/L, DAP= daptomycin, CIP= ciprofloxacin, $MIC_{(DAP)}$= minimum inhibitory concentration of daptomycin, $MIC_{(CIP)}$= minimum inhibitory concentration of ciprofloxacin, ND= not determined*

The only four concentration ratios, where inhibition of bacterial growth was detected, were included in the evaluation (see Table 18). Three combinations have FIC index values close to value 1, indicating additive effect, and the remaining combination indicates indifference.

Table 18: *Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of daptomycin and vancomycin. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, DAP= daptomycin, CIP= ciprofloxacin, FIC(A)= fractional inhibitory concentration of daptomycin, FIC(B)= fractional inhibitory concentration of ciprofloxacin*

MIC (DAP : CIP) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
0.125 : 0.5	1.0078125	indifference, near to additive effect
0.25 : 0.5	1.015625	indifference, near to additive effect
0.5 : 0.5	1.0625	indifference, near to additive effect
4 : 0.5	1.25	indifference

Evaluation of the DAP+RIF combinations is similar to the evaluation of the DAP+LIN combinations— DAP does not increase the efficacy of RIF (see Figure 24).

Figure 24: *Heat map of checkerboard assay of daptomycin and rifampicin combination. Heat plot describing the antibacterial activity of daptomycin and rifampicin in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Green dotted lines describe the MIC of antibiotics. Legend: MIC(DAP)= 16 mg/L, MIC(RIF)= 0.005 mg/L, DAP= daptomycin, RIF= rifampicin, MIC(DAP)= minimum inhibitory concentration of daptomycin, MIC(RIF)= minimum inhibitory concentration of rifampicin, ND= not determined*

In parallel to the DAP+LIN combinations, four FIC index values for selected DAP+RIF combinations were close to 1, and two values indicate indifference (see Table 19).

Table 19: *Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of daptomycin and vancomycin. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, DAP= daptomycin, RIF= rifampicin, FIC(A)= fractional inhibitory concentration of daptomycin, FIC(B)= fractional inhibitory concentration of rifampicin*

MIC (DAP : RIF) mg/L	$\sum$FIC = FIC (A) + FIC (B)	Interpretation
0.125 : 0.005	1.0078125	indifference, near to additive effect
0.25 : 0.005	1.015625	indifference, near to additive effect
0.5 : 0.005	1.03125	indifference, near to additive effect
1.0 : 0.005	1.0625	indifference, near to additive effect
2.0 : 0.005	1.125	indifference
4.0 : 0.005	1.25	indifference

5.12. Evaluation of the efficacy of tigecycline and vancomycin, tigecycline and linezolid, and tigecycline and ciprofloxacin, in combination

For the preparation of TIG+VAN drug combinations, solutions with different concentrations of VAN in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), and solutions of TIG were pipetted in a horizontal direction (six rows). For the preparation of TIG+LIN and TIG+CIP drug combinations, solutions with different concentrations of TIG in CAMHB were pipetted into wells of 96-well microtitre plate in a vertical direction (six columns), solutions of LIN and CIP were pipetted in a horizontal direction (six rows) (see Figure 7). Further, both antibiotics were pipetted separately, and lastly, one row of six wells was singled out for positive control. Subsequently, 10 µl per well of the bacterial inoculum was inoculated into wells.

After a 24-hour incubation period at 37°C, an evaluation of the inhibition of growth by the naked eye was done, and absorbance was measured at wavelength 530 nm with a multi-mode plate reader. MIC of individual antibiotics for MRSA, ATCC 43300 strain was determined as $MIC_{(VAN)}$= 2 mg/L, $MIC_{(LIN)}$= 4 mg/L, $MIC_{(CIP)}$= 0.5 mg/L. Similarly to DAP, in all combinations, the MIC of TIG could not be determined, ($MIC_{(TIG)}$> 0.25 mg/L). MIC of VAN and CIP within the checkerboard assay corresponded to the same value as determined in Chapter 4.1, MIC of LIN was lower than the determined value (dropped from 8 mg/L to 4 mg/L), and MIC of TIG was higher than the determined value (see Table 2). According to the breakpoints in EUCAST, the MRSA ATCC 43300 strain based on these results is recognized as susceptible to all above-mentioned antibiotics, with unknown susceptibility to TIG (see Table 1). Comparing the determined MIC value (0.00625 mg/L) (see Table 2) and the EUCAST MIC value (0.5 mg/L) (see Table 1) of TIG, it can be seen that the reference value is 3-fold lower than the EUCAST value. As the MIC of tigecycline could not be determined accurately, the efficacy of combinations with TIG could not be properly evaluated, and subsequently, TIG was removed from further testing.

The evaluation of these combinations was done analogically to the evaluation of combinations of DAP with other antibiotic agents (see Chapter 5.11). The $MIC_{(TIG)}$ was assumed to be 0.5 mg/L, and the FIC index values were calculated using the equation in Figure 10, providing an approximate evaluation of the efficacy of these combinations (see Table 20, Table 21, and Table 22. Heat maps of each combination (Figure 25, Figure 26, and Figure 27) were created to assess the efficacy of combinations with concentrations of TIG lower than assumed $MIC_{(TIG)}$.

The heat map of the TIG+VAN combination (Figure 25) demonstrates that except for one combination (TIG:VAN, 0.00195312:2 mg/L), in combinations with the final concentration of VAN corresponding to $MIC_{(VAN)}$ acting alone (2 mg/L) the inhibition of the bacterial growth was registered. It should be noted that in two combinations with the final concentration corresponding to sub-inhibitory $MIC_{(VAN)}$, the potentiation of bacterial growth was registered (TIG:VAN, 0.03125:1 mg/L, 0.015625:1 mg/L).


TIG (mg/L)	0.0625	0.125	0.25	0.5	1	2	VAN (mg/L)
0.5	ND	ND	ND	ND	ND	ND	
0.25	ND	ND	ND	ND	ND	ND	
0.125	ND	ND	ND	ND	ND	ND	
0.0625	3.750997606	19.792498	9.736632083	16.67996808	15.2434158		
0.03125	8.778930567	26.97552594	19.55307263	26.01755786	-51.31683958		
0.015625	10.45490822	22.42617717	11.89146049	11.89146049	-58.97845172		
0.0078125	19.07422187	26.49640862	19.792498	26.25698325	34.15802075		
0.00390625	19.07422187	11.89146049	35.35514765	32.4820431	15.72226656		
0.00195312	24.34158021	31.76376696	24.5810059	26.97552594	0.877893057	45.17758819	

Figure 25: *Heat map of checkerboard assay of tigecycline and vancomycin combination. Heat plot describing the antibacterial activity of tigecycline and vancomycin in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Boxes in grey were not taken into consideration for evaluation (an error occurred during preparation). Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(TIG)}$= 0.5 mg/L, $MIC_{(VAN)}$= 1 mg/L, TIG= tigecycline, VAN= vancomycin, $MIC_{(TIG)}$= minimum inhibitory concentration of tigecycline, $MIC_{(VAN)}$= minimum inhibitory concentration of vancomycin, ND= not determined*

Five combinations of TIG+VAN, where inhibition of bacterial growth was registered by the naked eye, were evaluated, and their FIC indexes were calculated. Four combinations have an FIC index value close to 1, near to additive effect (see Table 20).

Table 20: *Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of tigecycline and vancomycin. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, TIG= tigecycline, VAN= vancomycin, FIC(A)= fractional inhibitory concentration of tigecycline, FIC(B)= fractional inhibitory concentration of vancomycin*

MIC (TIG : VAN) mg/L	$\sum$FIC = FIC (A) + FIC (B)	Interpretation
0.0625 : 2	1.125	indifference
0.03125 : 2	1.0625	indifference, near to additive effect
0.015625 : 2	1.03125	indifference, near to additive effect
0.0078125 : 2	1.015625	indifference, near to additive effect
0.00390625 : 2	1.0078125	indifference, near to additive effect

From Figure 26 can be seen that TIG does not seem to increase the efficacy of LIN. However, in combination with the concentration of LIN corresponding to its ½ MIC sub-inhibitory concentration, the percentage of inhibition values were above 50%.
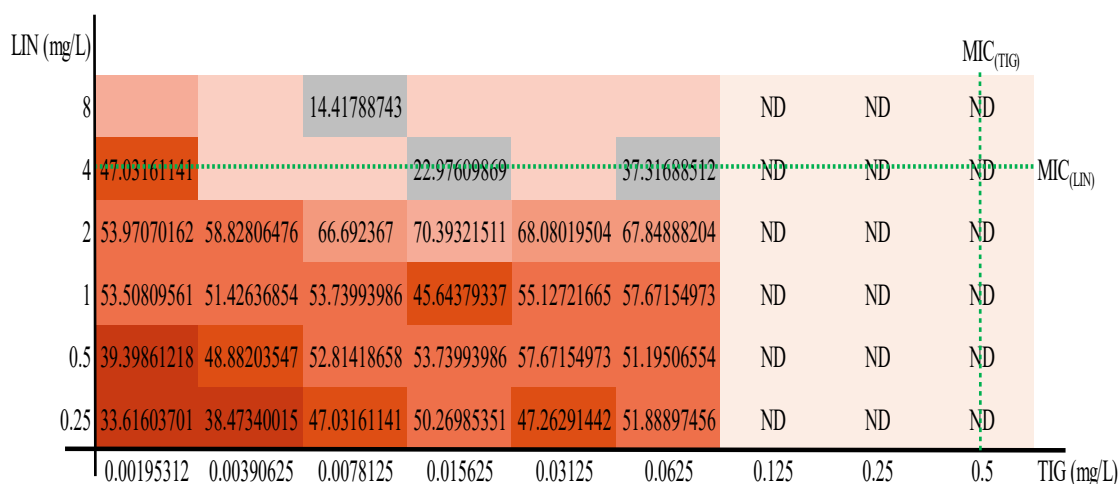
Figure 26*: Heat map of checkerboard assay of tigecycline and linezolid combination. Heat plot describing the antibacterial activity of tigecycline and linezolid in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Boxes in grey were not taken into consideration for evaluation (an error occurred during preparation). Green dotted lines describe the MIC of antibiotics. Legend: MIC(TIG)= 0.5 mg/L, MIC(LIN)= 4 mg/L, TIG= tigecycline, LIN= linezolid, MIC(TIG)= minimum inhibitory concentration of tigecycline, MIC(LIN)= minimum inhibitory concentration of linezolid, ND= not determined*

The FIC index values of three TIG+LIN combinations with the concentration of LIN corresponding to MIC$_{(LIN)}$ (2 mg/L) were calculated, and the evaluation of interaction was that of near additive effect (see Table 21).

Table 21*: Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of daptomycin and linezolid. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, DAP= daptomycin, LIN= linezolid FIC(A)= fractional inhibitory concentration of daptomycin, FIC(B)= fractional inhibitory concentration of linezolid*

MIC (TIG : LIN) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
0.03125 : 4	1.0625	indifference, near to additive effect
0.0078125 : 4	1.015625	indifference, near to additive effect
0.00390625 : 4	1.0078125	indifference, near to additive effect

The heat map of the TIG+CIP combination (Figure 27) shows that in all tested combinations with ½ MIC sub-inhibitory concentration of CIP, the percentage of inhibition values were above 50%. However, CIP was not potentiated by TIG.
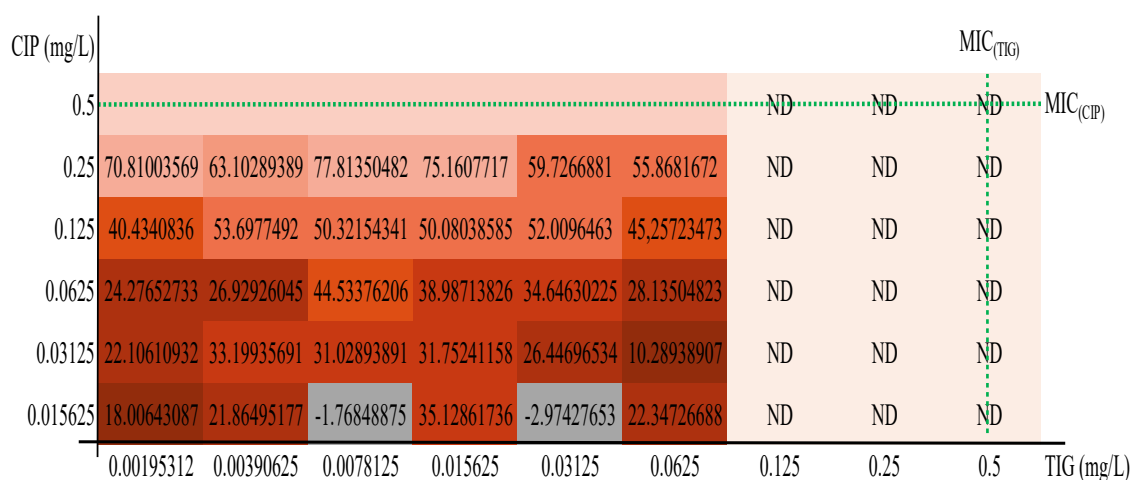
Figure 27: Heat map of checkerboard assay of tigecycline and ciprofloxacin combination. Heat plot describing the antibacterial activity of daptomycin and ciprofloxacin in combination against Staphylococcus aureus, ATCC 43300 strain. Boxes with value represent wells with bacterial growth detected. Boxes with no values represent wells with no presence of bacterial growth. Boxes in grey were not taken into consideration for evaluation (an error occurred during preparation). Green dotted lines describe the MIC of antibiotics. Legend: $MIC_{(TIG)}$= 0.5 mg/L, $MIC_{(CIP)}$= 0.5 mg/L, TIG= tigecycline, CIP= ciprofloxacin, $MIC_{(TIG)}$= minimum inhibitory concentration of tigecycline, $MIC_{(CIP)}$= minimum inhibitory concentration of ciprofloxacin, ND= not determined

Six combinations of TIG+CIP, where inhibition of bacterial growth was registered, were further evaluated, and their FIC index was calculated. In five combinations, FIC index values were close to 1, indicating an additive effect (see Table 22).

Table 22: Total fractional inhibitory concentration (FIC) indexes and results interpretation of mutual interaction of tigecycline and ciprofloxacin. Legend: MIC= minimum inhibitory concentration, FIC= fractional inhibitory concentration, TIG= tigecycline, CIP= ciprofloxacin, FIC(A)= fractional inhibitory concentration of tigecycline, FIC(B)= fractional inhibitory concentration of ciprofloxacin

MIC (TIG : CIP) mg/L	∑FIC = FIC (A) + FIC (B)	Interpretation
0.0625 : 0.5	1.125	indifference
0.03125 : 0.5	1.0625	indifference, near to additive effect
0.015625 : 0.5	1.03125	indifference, near to additive effect
0.0078125 : 0.5	1.015625	indifference, near to additive effect
0.00390625 : 0.5	1.0078125	indifference, near to additive effect
0.00195312 : 0.5	1.00390624	indifference, near to additive effect

6. DISCUSSION

The infections caused by antibiotic-resistant *S. aureus* have accumulated in severity throughout the years. MRSA can appear in both hospital and community environments and cause several infections, ranging from mild skin infections to severe infections like endocarditis, deep tissue infections, or infections associated with invasive medical devices (joint prosthetic, valve prosthetic, *etc*.) (78). In addition to MRSA, although rare, the emergence of VISA, hVISA, VRSA, and DNSA carries significant clinical concern. In conclusion, considering these facts, the need for appropriate combat strategies increases (79).

There have been several studies demonstrating *S. aureus* resistance to various antibiotic classes: β-lactams, glycopeptides, quinolones, tetracyclines, rifampicin, cotrimoxazole, and others. The current first-line antibiotics for MRSA infections are vancomycin, daptomycin, and linezolid. Vancomycin is a glycopeptide that inhibits the synthesis of bacterial wall and is the first choice antibiotic for the treatment of infections caused by MRSA. However, some limitations are associated with this drug, such as slow killing time, poor tissue penetration, and duration-dependent nephrotoxicity. Daptomycin, a cyclic lipopeptide with efficacy similar to vancomycin, cannot be used for pulmonary infections, and linezolid, an oxazolidinone that inhibits protein synthesis by binding on the 23S ribosomal subunit, has several significant side effects when administrated for a prolonged period. (56)

To improve the outcome of antibiotic treatment, a second antibiotic agent can be added to the first-line agent, like β-lactams, rifampicin, ciprofloxacin, tetracyclines, aminoglycosides, and others (79). Several studies have been conducted to determine the activity and efficacy of many antibiotic combinations, mainly by *in vitro* testing or by *in vivo* animal testing. Recently, there has been an increase in randomized controlled trials for the combination therapy of *S. aureus* infections. However, the results of these studies are not uniform, and the efficacy of combination therapy remains in some aspects controversial. (61; 80)

In this study, seven commercially available antibiotics and their pair-wise combinations were evaluated to determine their activity against MRSA*,* ATCC 43300 strain. The checkerboard microtitrate method was employed to test combinations of selected antibiotics with different concentration ratios. For evaluation, the inspection of the growth by the naked eye, together with spectrophotometric measurement, was included in each assay.

Measured data were processed, and the percentage of partial inhibition of the bacterial growth, compared to positive controls (bacteria unexposed to antibiotic drugs) was calculated. Subsequently, heat maps were created, and the FIC index was calculated to determine the nature of the mutual interaction of selected drugs in pair-wise combinations. In this thesis, the interpretation of the calculated FIC index values was as follows: FIC> 4 indicating antagonism, FIC> 1–4 indicating indifference, FIC= 0.5–1 indicating additive effect, and FIC< 0.5 indicating synergy. This classification is the same as the one proposed by the *Journal of Antimicrobial Chemotherapy* (81). It is important to note that the interpretation of FIC index values may vary according to different authors.

Altogether, seventeen combinations were tested in 6×6 mode— for each combination, thirty-six possible sub-combinations at different target concentration ratios were tested. Out of all tested antibiotic combinations and sub-combinations, one combination expressed an antagonistic effect; two combinations expressed indifference bordering on antagonism; ten combinations expressed an indifferent effect; two combinations showed mostly indifference except for sub-combinations, where the additive effect was registered; 2 combinations expressed additive effect. Among combinations that displayed indifference are sub-combinations with FIC index values close to 1 (i.e. after rounding, the FIC value would be 1)— near to the additive effect. One combination that displayed an additive effect has one sub-combination with an FIC index close to 0.5 (after rounding)— near to the synergic effect.

In all evaluated combinations of two first-line antibiotics, VAN+LIN, the indifferent effect was revealed. In the reviewed literature, most studies evaluating this combination using the checkerboard method (82; 83) and the time-kill method (84) report indifference or even slight antagonism (85; 86). Only in one study, the additive effect of this combination, evaluated by the E-test method was recognized (87). In conclusion, our results are in agreement with published results.

Similarly, the outcomes from our *in vitro* evaluation of the combination VAN+CIP report indifferent effects. Nevertheless, if the FIC index for three different VAN+CIP concentration ratios values were to be rounded, the additive effect can be mentioned in these combinations. Some studies focused on this combination have been published. Namely, in the study of authors Gradelski, *et al.* (2001) (88), the time-kill assay, and methicillin-susceptible strain, *S. aureus* ATCC 29213 were employed. Within this study, no effect of VAN on the activity of CIP was detected. The *in vivo* study of the combination therapy with CIP and VAN is available as well (89), with the conclusion being indifference. However, in the case of employment of VISA and hVISA strains, this combination, evaluated using a time-kill assay, showed synergy, regardless of the strain's susceptibility to ciprofloxacin (90). In another study published by Kamble, *et al.* (2022) (91), the checkerboard assay and time-kill assay were employed. In this study, the synergistic effect of this combination, as well as a decrease in the number of surviving cells using time-kill assay, together with the ability to disrupt biofilm consortia and reduce the presence of persistent bacterial cells in biofilms were revealed.

While the combination of VAN+COT showed mostly indifference, the concentration ratio VAN:COT, 1:0.5 mg/L displayed additive potential. The *in vitro* studies evaluating this combination are scarce. One study reports a synergistic effect of this combination using a time-kill assay (92), and another reports either synergy or additive effect using an E-test method (87).

The evaluation of the efficacy VAN+RIF combination has been the subject of many published studies. Rifampicin, despite the high frequency of rifampicin resistance in bacteria, is a very attractive antibiotic for combination therapy thanks to its bactericidal effect, good tissue penetration, accumulation in cerebrospinal fluid, and activity against biofilms (93). Despite the advantages that rifampicin has to offer, the efficacy of the VAN+RIF combination appears to be controversial. The reviewed literature shows a disparity in used *in vitro* testing methods, results interpretation, as well as in outcomes using the same testing method (57; 94; 95). A huge, multi-center, randomized, double-blinded, placebo-controlled trial was conducted in the United Kingdom to determine the efficacy of rifampicin as an adjuvant drug to standard therapy for *S. aureus* infections (ARREST trial, https://doi.org/10.1016/S0140-6736(17)32456-X). While this trial yielded a lot of important information, and the conclusion was that combination with rifampicin did not show any significant advantage compared to monotherapy, it is important to note that this is most likely only applicable for MSSA infections, as this trial did not separate patients with MSSA from patients MRSA infections. Only 6% of patients (47 out of 758) had MRSA infections. (96) The use of the VAN + RIF combination is recommended only for MRSA infective endocarditis involving prosthetic valves or other prosthetics by the American Hearth Association (97) and European Society of Cardiology (98).

The studies evaluating the combination LIN+CIP report no synergistic effect, whether the time-kill method (85), the checkerboard method (82), or the E-test (99) was used. To conclude, our results are in agreement with published results.

There is a limited number of published studies focused on the evaluation of the inhibitory potential of the LIN+COT combinations. To appoint at least one – in the study published by Kaka, *et al*. (2006) (100), the efficacy of the LIN+COT combination was evaluated by the time-kill method, and the combination does not display any significant potential. Likewise, in our study, no significant benefit (synergistic effect) from the LIN+COT combination was revealed.

In published studies evaluating the efficacy of the combination LIN+RIF, indifference is reported either using the time-kill method (85), the checkerboard method (82), or the E-test method (95; 101). In another study published by Baldoni, *et al.* (2009) (102), the time-kill assay, together with an *in vivo* guinea pig model, were employed. In addition, this study was focused on the development of rifampicin resistance. According to the obtained results, authors conclude that LIN+RIF combination represents an option for implant-associated infections caused by quinolone-resistant *S. aureus* strains. In the study published by Jacqueline, *et al.* (2003) (86), the interaction of LIN combined with RIF led to the additive interaction and the inhibition of rifampicin-resistant bacteria. The outcome of *in vitro* testing in this thesis is conclusive with the revised literature— overall, the combination displayed indifference, except for the combination at concentration ratio LIN:RIF, 2:0.00125 mg/L showing additive effect.

For the CIP+COT combination, only a limited number of studies have been published. The additive effect was recognized for this combination in the study published by Gosbell (2006) (103), and the synergistic effect in the study published by Kang, *et al*. (2016) (90). In both studies, the time-kill method was used. However, within our study, only an indifferent effect was recognized.

There are inconsistent conclusions regarding to impact of the CIP+RIF combination. While few published studies report the synergy using the time-kill method (92), others report no synergistic potential using the checkerboard method (88), the time-kill method (88), or the E-test method (101). The systematic review of the literature conducted by Perloth, *et al*. (2007) (94) mostly reports indifference or antagonism of this combination. The evaluation of the CIP + RIF combination in this thesis does not bring conclusive verification to either of the described outcomes— it was determined by this study that ciprofloxacin potentiates the antibacterial effect of rifampicin, and the additive effect of four concentration ratios was recognized.

In this thesis, the combination COT+RIF was the only one that displayed additive potential, nearing synergy in concentration ratio COT:RIF, 0.03125:0.005 mg/L. However, the published studies show inconsistent results. All reviewed sources report either antagonism using the time-kill method (100), indifference using the E-test method (101), or synergy using the disk diffusion test (104). Despite the results disparity of *in vitro* testing, this combination seems to display promising results from clinical trials, especially for the treatment of osteomyelitis and deep-seated soft tissue infections (105; 106).

A combination of DAP+VAN does not appear to be beneficial. There is a concern about the cross-resistance appearance between vancomycin and daptomycin— reduced vancomycin susceptibility, caused by a mutation on the *rpo*B gene, may be a cause for the reduction of daptomycin susceptibility in *S. aureus* (107; 108). The study conducted by Tsuji *et al*. (2005) (95) evaluated this combination by using an E-test or time-kill method, and indifference or additivity was registered (95). Other studies report an additive effect (using the E-test method) (87; 109).

Evaluation of the DAP+LIN combination using the pharmacokinetic/ pharmacodynamic model showed better efficacy than either of the antibiotic agents alone (110). In the review done by Antonello *et al.* (2022) (109), the evaluation of this combination reports mostly indifference, using either the checkerboard method, the time-kill method, or the pharmacokinetic/ pharmacodynamic model. This correlates with the results obtained in this thesis. However, two studies, using the checkerboard method registered mostly additive effect or synergy (111; 112), and one study, using the time-kill method, presented an antagonistic effect of this combination (113).

In this thesis, the combination DAP+CIP did not show a promising effect at the sub-inhibitory concentration of daptomycin, as opposed to a study conducted by Kamble *et al.* (2022) (91), which reports synergy using the same *in vitro* testing method.

The outcomes of the efficacy evaluation of the DAP+RIF combination are rather controversial. Interestingly, in the review conducted by Antonello, *et al.* (2022) (109), the *in vitro* tests of this combination showed either indifference or additive effect, while *in vivo* tests demonstrated synergy. This outcome is supported by findings in the review conducted by Nguyen, *et al.* (2009) (56). However, in the study conducted by Rose, *et al.* (2013) (114), a mostly synergistic effect of this combination using the checkerboard and the time-kill method is reported. It is important to note that the synergy was registered when the concentration of daptomycin was ½ MIC, and the loss of efficacy of the DAP+RIF combination was registered with the concentration of daptomycin of 4 MIC.

The combination of TIG+VAN seems like an attractive option, given the spectrum of activity and mode of action of tigecycline. However, *in vitro* tests carried out by the time-kill method (115) or checkerboard microdilution method (87; 116) pointed to an indifferent effect. These results are consistent with the outputs from experiments included in this thesis.

Similarly, the outcome of evaluation using the checkerboard method for combinations TIG+LIN (83; 117), and TIG+CIP (117) was indifference, which correlates with the results in this thesis.

The reliability of literature evaluating the outcome of *in vitro* tests, and the credibility of results in this thesis is not without limitations. These important factors create bias:

- the *in vitro* testing method used for the evaluation of antimicrobial drug activity— the results from the evaluation of drug efficacy using the checkerboard microdilution method are not comparable to results from the time-kill methodology (118). There are not many studies evaluating the efficacy of combinations of antibiotic drugs using the checkerboard microdilution method, which is why studies using the time-kill methodology and/or others were included.
- concentration ratios of the evaluated antibiotics in combination may vary
- differences in included tested *S. aureus* strain
- differences in the data interpretation— results from the same *in vitro* testing method may vary.

In summary, all these factors can play a decisive role in the final outputs of studies focused on the efficacy of drug combinations.

7. CONCLUSION

This study was conducted to find combinations of antibiotics that showed promising, preferably synergistic interactions. These combinations could be used for extensive and comprehensive studies, such as part of "anti-infective cocktails", especially acting against bacterial staphylococcal communities called biofilms.

The checkerboard microdilution method was employed for the evaluation of seventeen antibiotic combinations at different concentration ratios. The FIC index was used for the recognition of mutual antibiotic drug interactions.

Out of seventeen combinations, seven of them (VAN+TIG, VAN+DAP, DAP+LIN, DAP+CIP, DAP+RIF, LIN+TIG, and CIP+TIG) could not be properly included in the evaluation, because MIC of DAP and TIG could not be properly determined, and only approximate evaluation was done. As for the other ten combinations, six of them (LIN+VAN, CIP+VAN, RIF+VAN, LIN+CIP, LIN+COT, CIP+COT) showed indifference; two showed mostly indifference, except for one concentration ratio, where the combination showed additive effect (COT+VAN, LIN+ RIF); one combination showed additive interaction, where one antibiotic agent was potentiated by the other (CIP+RIF); and one combination showed additive interaction, where both antibiotics mutually potentiated each other (RIF+COT).

Combinations of CIP+RIF and RIF+COT are two pair-wise antibiotic combinations, which are recommended for further testing as part of drug cocktails.

2D combinations – summary:

 - VAN + LIN => indifference

 - VAN + CIP => indifference

 - VAN + COT=> indifference (concentration ratio 0.5:1 mg/L and 1:1 mg/L – additive effect)

 - VAN + RIF => indifference

 - LIN + CIP => indifference

 - LIN + COT => indifference

 - LIN + RIF => indifference (concentration ratio 2:0.00125 mg/L – additive effect)

 - CIP + COT => indifference

 - CIP + RIF => indifference, additive (RIF is potentiated by CIP)

 - COT + RIF => additive effect (mutual potentiation)

- DAP + VAN/LIN/CIP/RIF => could not be properly determined

 - TIG + VAN/LIN/CIP => could not be properly determined

8. LIST OF ABBREVIATIONS

ATCC 43300	American Type Culture Collection, collection number 43300
CAMHB	cation-adjusted Müller-Hinton Broth
CA-MRSA	community-acquired methicillin-resistant *Staphylococcus aureus*
CAP	community-acquired pneumonia
CDC	Centers for Disease Control and Prevention
CIP	ciprofloxacin
CNS	central nervous system
COT	cotrimoxazole
DAP	daptomycin
dfr(A/B/G/K)	dihydrofolate reductase (A/B/G/K) gene
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DHP	dihydropteroate
DHPS	dihydropteroate synthase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNSA	daptomycin-nonsusceptible *Staphylococcus aureus*
ECDC	The European Centre for Disease Prevention and Control
EMA	European Medicines Agency
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FIC	fractional inhibitory concentration
HA-MRSA	hospital-acquired methicillin-resistant *Staphylococcus aureus*
HAP	hospital-associated pneumonia
HIV	human immunodeficiency virus
LA-MRSA	livestock-associated methicillin-resistant *Staphylococcus aureus*

LIN	linezolid
MDR	multidrug resistance/resistant
mec(A)	methicillin-resistant genetic component (A)
*mep*A/R	multidrug export protein A/R
MHA	Müller-Hinton agar
MHB	Müller-Hinton broth
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-susceptible *Staphylococcus aureus*
OPAT	outpatient parental antimicrobial therapy
PABA	para-aminobenzonic acid
PAE	post-antibiotic effect
PAE-SME	postantibiotic sub-MIC effect
PBP	penicillin-binding protein
PDR	pan-drug-resistant
RIF	rifampicin
RNA	ribonucleic acid
*rpo*B	RNA polymerase B
rRNA	ribosomal ribonucleic acid
S. aureus	*Staphylococcus aureus*
SAB	*Staphylococcus aureus* bacteremia
SCC*mec*	staphylococcal cassette chromosome
SSTI	skin and soft tissue infection
tet(A/K/L/M/O)	tetracycline-resistance protein (A/K/L/M/O)
THF	tetrahydrofolate
TIG	tigecycline
tRNA	transfer ribonucleic acid
VAN	vancomycin
van	vancomycin-resistance gene

VAP	ventilator-associated pneumonia
VISA	vancomycin-intermediate *Staphylococcus Aureus*
VRSA	vancomycin-resistant *Staphylococcus Aureus*
VSSA	vancomycin-susceptible *Staphylococcus Aureus*
WHO	World Health Organization
XDR	extensively drug-resistant

9. LIST OF FIGURES

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