The Impact of Phytochemicals on Antibiotic-Resistant Microbiota and Advanced Biotyping on Multidrug-Resistant Bacteria

Master's Thesis

Sami Shajarat
Supervisors:
Allan Stensballe
Thomas Bouet Guldbæk Poulsen

A thesis presented for the degree of
Master's in Biomedicine

AALBORG
UNIVERSITY

Translational Biomarkers for Pain and Precision Medicine
Aalborg University
Aalborg, Denmark
June 2022

Abstract

Background: The occurrence of multidrug-resistant bacteria has led to one of the greatest challenges in public health care. Bacteria are continuously evolving resistance mechanisms, such as extended spectrum β-lactamases, which alters the effect of antibiotics and thereby limits treatment options. It is therefore crucial to find possible solutions in order to prevent further escalation. Factors such as antibiotic stewardship programs, new antibacterial agents, and faster antibiotic susceptibility testing are keynotes in the combat against these microorganisms.

Methodology: In this project, we evaluated the antibacterial capabilities of phytochemicals extracted from Salicornia Ramosissima obtained by aqueous or organic solvent based extraction. Bactericidal and bacteriostatic tests were performed using the agar well diffusion method and an optical density test. In addition, the potential of determining bacterial resistance proteins to identify resistance mechanisms using fast LC-MS/MS for novel antibiotic susceptibility testing by advanced biotyping was also assessed. This involved extraction of bacterial proteins with S-trap based in-situ proteolysis and fast analysis through the Fast LC-MS/MS analysis coupled to ion-mobility tandem mass spectrometry.

Results: We did not observe any bactericidal or bacteriostatic effect from the phytochemicals extracted from Salicornia Ramosissima at the available concentrations of bioactive compounds. For the advanced biotyping, we were, however, able to identify 30 resistance proteins using LC-MS/MS, three of which (TEM-55, SHV-65, and CTX-M-82) are related to ESBL.

Conclusion: In this study, we can conclude that the concentration of phytochemicals needs to be higher before an antibacterial effect is possible. However, LC-MS/MS demonstrated promising results in determining resistance proteins and the potential of incorporating this system in AST should further analysed.

Resume

Baggrund: Udviklingen af multi-resistente bakterier er blevet en af de største problematikker for den offentlige sundhedssektor. Bakterier udvikler resistens mekanismer, som udvidet spektrum β-laktamase, der forhindrer virkningen af antibiotika og derved begrænser behandlingsmulighederne. Af denne årsag er det vigtigt at finde løsninger, der kan forhindre yderligere eskalering. Her er faktorer som, antiobiotisk stewardship programmer, nye antibakterielle midler og hurtigere identifisering af antibiotisk resistens vigtige nølgepunkter i kampen mod disse mikroogranismer.

Metode: I dette studie, testede vi antibakterielle egenskaber af fytokemikalier fra Salicornia Ramosissima, udtaget enten vandligt eller organisk. Den baktericide og bakteriostatiske virkning blev testet ved hjælp af agar brønd diffundering og en optisk-tæthed test. Ydermere, analyserede vi potentialet i at analysere bakterielle resistens proteiner til at identificere resistens mekanismer ved brug af LC-MS/MS. Dette involverede udtrækning af bakterielle proteiner ved hjælp af S-trap baseret in-situ proteolyse og hurtig analyse ved hjælp af hurtig LC-MS/MS analysis tilkoblet ion mobilitet tandem masse spektrometri.

Resultater: Vi observerede ingen baktercid eller bakteriostatisk effekt fra Salicornia Ramosissima ekstraktet med den mulige koncentration af bioaktive stoffer. Dog i forhold til den advancerede biotyping, identificerede vi 30 resistens proteiner ved brug af LC-MS/MS, hvoraf tre (TEM-55, SHV-65 og CTX-M-82) er relaterede til ESBL.

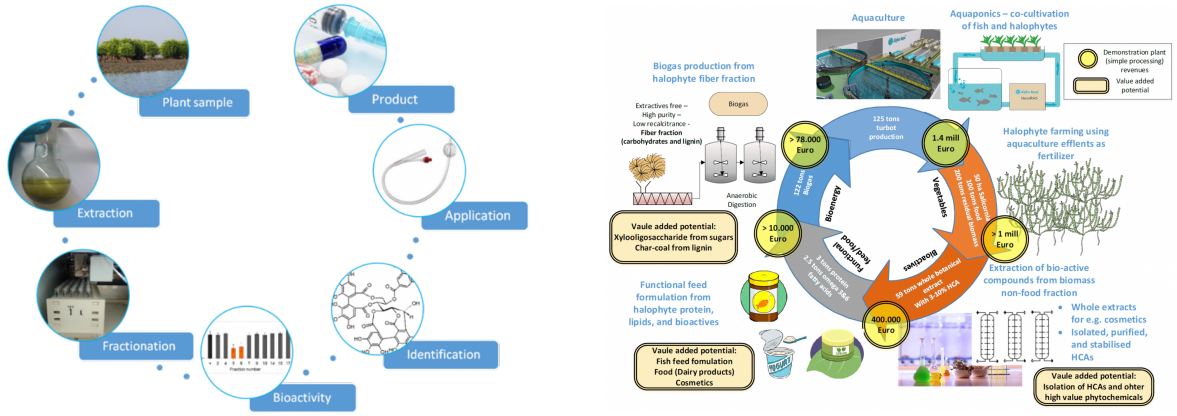
Konklusion: Vi kan i dette studie konkludere at koncentrationen af fytokemikalier skal være højere for at få en antibakteriel effekt. Dog vidste LC-MS/MS lovende resultater i forbindelse med identificering af resistens proteiner og denne metodes potentiale i AST bør derfor yderligere analyseres.

Abbreviation list

A-site = Acceptor site
ARB = Antibiotic resistant bacteria
AST = Antibiotic susceptibility testing
AUC = Area under the curve
DDA = Data-dependent acquisition
DNA III HE = DNA polymerase III holoenzyme
E. coli = Escherichia coli
E-site = Exit site
ESBL = Extended-spectrum-beta-lactamases
FD = Freeze-dried
K. pneumonia = Klebsiella pneumonia
LB = Lysogeny broth
LC-MS/MS = Liquid chromatography tandem mass spectrometry
MALDI-TOF MS = Matrix-assisted laser-desorption/ionization time of flight mass spectrometry
MBC = Minimum bactericidal concentration
MIC = Minimum inhibitory concentration
OM = Outer membrane
P-site = Peptidyl site
PASEF = Parallel-acquisition series fragmentation
PBP = Penicillin binding protein
PP = Patent-protected
S. aureus = Staphylococcus aureus
S. pneumonia = Streptococcus pneumonia
SD = Spray-dried
SEDS = Shape, elongation, division, and sporulation
TIMS = Trapped ion mobility spectrometry
TOF = Time-of-flight

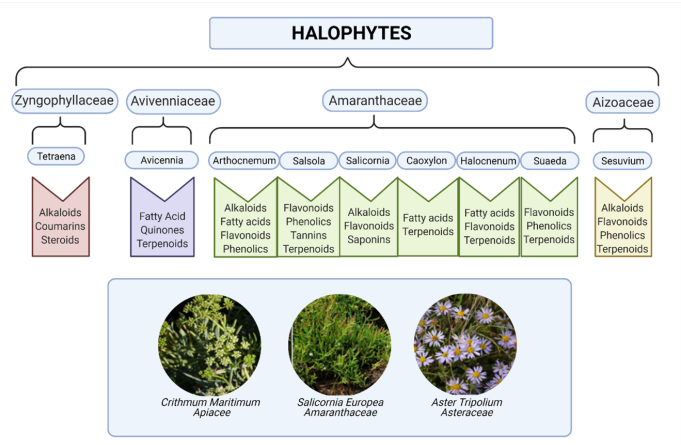
The AquaCombine project

With the world's population increasing as well as decreasing farmlands, there is a need for sustainability, particularly in relation to soil salinity. This includes focusing on the production of food and bio-products derived from sustainably produced biomass. As part of the EU Horizon 2020 program, the AQUACOMBINE project (www.aquacombine.eu) attempts to utilize the complete produced biomass by combining aquaculture and farming of halophytes. This means that by using halophyte plants and the principles of circular economy the usage of residues to create new products is increased, thus avoiding any waste. This in turn increases both the internal value and sustainability. An overview of the AquaCombine work cycle is depicted in the figure below



Flowchart illustrating the circular economy and applications for bioactive plant extracts for biomedical applications, including as source of potential antimicrobial compounds - Pictures from https://www.aquacombine.eu/?page_id = 147 and https://www.aquacombine.eu/?p=933

Halophytes are species with the capability of growing and complete a lifecycle in a salt concentration of at least 200mM. This ability might be related to their production of bioactive compounds, including free radical scavenging secondary metabolites. In AquaCombine to halophytic sources are assessed namely the *Salicornia Europea* and *Salicornia Ramosissima* and due to their high content of secondary bioactive metabolites such as, saponins, flavonoids, alkaloids, betacyanines, and isoflavonoids, these plant displays several health promoting properties, which are particularly related to anti-inflammatory, antioxidative, antitumor, and antimicrobial.



Phytochemical composition of some common coastal halophytes - Created with BioRender

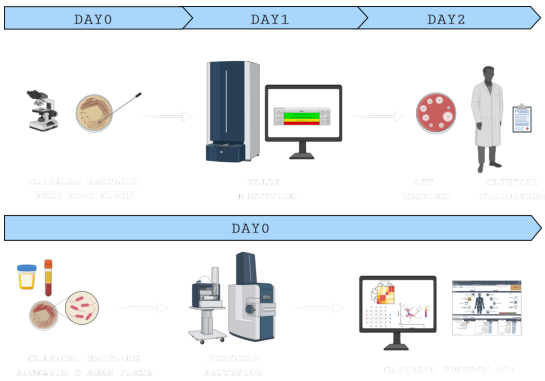
Studies have previously demonstrated antibacterial properties of the Salicornia family (Essaidi et al. 2013). The study demonstrated that the extract, from *S. Herbacae*, mostly affected Gram-positive bacteria as compared to Gram-negative. These results are suggested to be attributed with multiple compounds from *S. Herbacae*. Especially, polyphenols are thought to be associated with the antimicrobial activity, as these along with other compounds are suggested to be potential cytochrome P450 enzyme inhibitors (Essaidi et al. 2013).

Background

The occurrence of multidrug-resistant bacteria has led to one of the greatest challenges in public health care. The "post-antibiotic" era is not far from becoming a reality, as antibiotics are continuously being defeated by bacteria. This opposes a great challenge as approximately 4.9 mio people died yearly as a part of antimicrobial resistance (Thompson 2022). This could be a result of initial antibiotic therapy failure, as a study by Serafim et al. describes that failure of controlling systemic infections within the 24-48 hours increases the risk of mortality and a study by Peeters et al. estimated that the initial antibiotic therapy failure was >60% across all countries. This and the fact that an extensive review of antibiotic-resistant bacteria (ARB) estimated that by the year 2050 ten million people's lives would be at risk yearly, due to the increase in drug-resistant infections, emphasize the need for controlling and identifying infectious microorganisms (O'Neill 2016; *Challenges to Tackling Antimicrobial Resistance* 2019; *Landscape of diagnostics against antibacterial resistance, gaps and priorities* 2019; Peeters et al. 2018; Serafim et al. 2020). Currently, Matrix-Assisted Laser-Desorption/Ionisation – Time of Flight Mass Spectrometry (MALDI-TOF MS) has become an accepted standard approach for bacterial identification as it, and the golden standard for antibiotic susceptibility testing (AST) analysis remains the disk diffusion method. Thus, it is crucial to obtain an understanding of the resistance mechanisms and possible solutions.
Klebsiella pnuemoniae, Streptococcus pneumoniae, Escherichia coli and *Staphylococcus Aureus* remain among some of the major concerns for the public health care, as they tend to cause nosocomial outbreaks and their broad range of antibiotic resistance limits treatment options (Alvarez, Labarca, and Salles 2010; Opoku-Temeng, Kobayashi, and DeLeo 2019; Jubeh, Breijyeh, and Karaman 2020; MacKinnon et al. 2020). Bacteria are able to inactivate antibiotics through several defence mechanisms, one of which includes producing β-lactamase to hydrolyse β-lactams and alter the efficiency of these drugs (Worthington and Melander 2013) New antimicrobial agents are therefore needed and with the long known history of treating infections with plants, phytochemicals has gained a lot of attention in the pursuit of new antibiotics (Barbieri et al. 2017; Essaidi et al. 2013).

One of the major causes to ARB is lack of antibiotic stewardship. Antibiotic stewardship aims to prevent overuse or misuse of antibiotics, which is especially seen in countries where antibiotics are sold unprescribed. Thus, it is crucial to monitor inappropriate or unnecessary antibiotic consumption to limit the occurrence of ARB. Improving antibiotic susceptibility testing (AST) would enhance this intervention, as the current AST method is time-consuming, resulting in empiric treatment until resistance data is available. Changing the turnaround time to same-day results could, therefore, have a positive impact on the overuse and misuse of antibiotics (J, F, and Cannady 2021; Rentschler, Kaiser, and Deigner 2021) Improving and discovering new rapid diagnostic methods of the bacterial resistance mechanism have therefore been promoted as it will quicken the initiation of treatment with the correct drug and improve clinical outcome (Habboush Y and Guzman N 2021; *Challenges to Tackling Antimicrobial Resistance* 2019).
To investigate this, we used liquid chromatography tandem mass spectrometry (LC-MS/MS).

Here, we set up a deep proteome investigation to identify strains related to resistance mechanisms and the time-needed for identification (Dimard E Foudraine et al. 2021). For an overview of the current method and proposed set-up refer to the figure below



An overview of the current and proposed workflow for baterial analysis using MS - Created using BioRender

The following introduction will describe the bacterial structure, resistance mechanisms, and the use of antibiotics for eliminating pathogenic bacteria. Next, the antibacterial potential of phytochemicals will be described, followed by a technological overview of how bacteria and susceptibility is identified. Lastly, a description of LC-MS/MS's potential in replacing current methods will be given.

Contents

1 Introduction

In order to obtain a better understanding on how antibiotics and potentially phytochemicals affects the bacteria, an understanding of their components and structure is needed. A bacterium consists of seven important components. (1) Cell envelope, (2) cytoplasm, (3) nucleoid, (4) plasmids, (5) inclusion bodies, (6) flagella, and (7) pili and fimbriae (M. Manisha 2021). The focus of this report will be on the cell envelope, cytoplasm, and plasmids, due to their role as antibiotic targets and in resistance development.

1.1 The Cell Envelope

The cell envelope is the membrane and other structures that surround and protect the cytoplasm and is crucial for the bacteria since they are exposed to various unpredictable environments. The gram staining developed by Christian Gram showed that the envelope differs between bacteria, leading to the classification we know today as gram-positive and gram-negative. In addition to its protective role, the cell envelope also filters nutrients and waste products from outside and inside the cell (Silhavy, Kahne, and Walker 2010).

1.1.1 Gram-negative Bacteria

The cell envelope of gram-negative bacteria is composed of three layers. (1) the outer membrane (OM), (2) a peptidoglycan cell wall, and (3) the cytoplasm (Silhavy, Kahne, and Walker 2010).

The OM is a lipid bilayer composed of glycolipids (mostly lipopolysaccharides) on the outside and phospholipids on the inside. The lipopolysaccharides play a major role in the functionality of the OM. The role of lipopolysaccharides is to transform the OM into an effective permeability barrier against small hydrophobic molecules, increasing the bacteria's resistance to antimicrobial compounds. This is the first important part of bacterial survival (Bertani and Ruiz 2018; Silhavy, Kahne, and Walker 2010).

The peptidoglycan wall determines, through its rigidity, the shape of the bacteria, which varies between cocci, rods, and comma. The peptidoglycan wall is composed of repeated N-acetyl glucosamine-N-acetyl muramic acid units crossed-linked by pentapeptide side chains. It is attached to the OM by murein lipoprotein, which embeds lipid into the OM (MRJ and KS 2021; Silhavy, Kahne, and Walker 2010).

The inner membrane is a phospholipid bilayer, which possess all the membrane proteins that functions as the membrane-associated organelles of eukaryotic cells. The functions include energy production, lipid biosynthesis, protein secretion, and transport (Silhavy, Kahne, and Walker 2010).

1.1.2 Gram-positive Bacteria

Unlike gram-negative bacteria, the OM is absent on the gram-positive bacteria. As a result, the gram-positive bacteria has a much thicker layer of peptidoglycan, supported with long anionic polymers, known as teichoic acids. The anionic effect of teichoic acid ensures cation homeostasis, which is crucial as it affects the rigidity and porosity of the cell wall and therefore the bacteria's susceptibility to antimicrobial components (Silhavy, Kahne, and Walker 2010).

1.2 The Bacterial Cytoplasm

The cytoplasm is a crowded polydisperse aqueous environment, consisting of several components including proteins, plasmids, and enzymatic megacomplexes. The bacteria rely on diffusion for

molecular transport and cytoplasmic mixing, due to the lack of cytoskeletal motor proteins seen in, e.g., eukaryotes. The diffusion therefore plays an essential part of bacterial life, as it determines the occurrence of molecular interactions and cell proliferation (Parry et al. 2014).

1.3 Bacterial Plasmids

Plasmids are circular or linear extrachromosomal replicons that constitute a substantial amount of the total genetic content of an organism. Plasmids can be introduced into a new host by various mechanisms, which allows genetic exchange in bacterial populations. This is a major contribution to the rapid evolution and adaptation abilities, which bacteria possess, especially in relation to antibiotic resistance. Many resistance genes are often present on a single plasmid, which allows the bacterium to efficiently transfer multiple resistance genes in a single conjugation event (Nikaido 2009; Shintani, Sanchez, and Kimbara 2015; Solar et al. 1998). Especially, resistance genes coding for enzymes, which modify or destroy antibiotics through inactivation, are often localized on plasmids. The plasmid-mediated enzyme extended-spectrum β lactamase (ESBL) is an example, as this plasmid also carries resistance genes against aminoglycosides, chloramphenicol, sulfonamides, trimethoprim, and tetracycline, which classifies ESBL producing bacteria as multidrug-resistant (A.M., M.M., and M. YU. 2018; Gupta et al. 2003). An overview of the bacterial components is depicted in Figure 1.

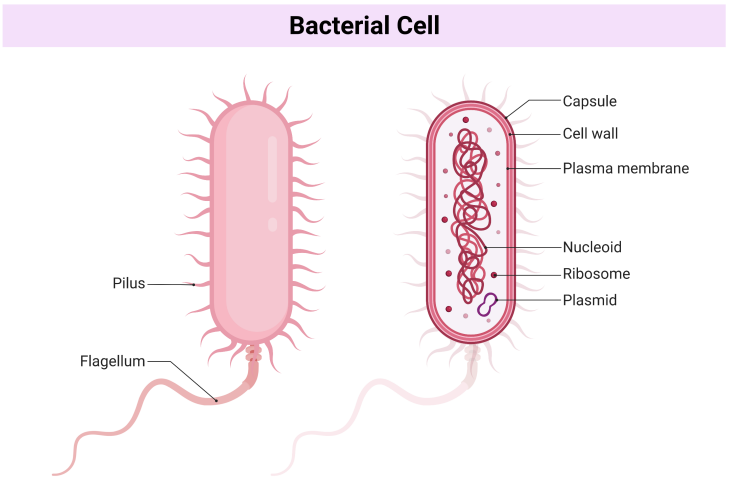


Figure 1: *An overview of the bacterial components - Created with Biorender*

1.4 Antibiotic Overview - Classification and Effects

Antibiotics are commonly used agents that weaken or kills infectious bacteria. Antibiotics exert their pharmacological effects through either blockage of cellular reproduction or by changing cellular function and processes. The pharmacological effect of antimicrobial agents is classified, either, as bactericidal or bacteriostatic. This is based on their in vitro effect, measured as the ratio between the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Calhoun, Wermuth, and Hall 2021). An overview of the two types of antibiotics is given in Table 1.

Category	Description	MBC to MIC ratio
Bactericidal	Kills bacteria	< 4
Bacteriostatic	Prevents bacterial growth	> 4

Table 1: *An overview of the antibiotic drug classification with description and criteria*

The MIC is determined by the lowest concentration that inhibits visible bacterial growth after 24 hours, whereas MBC is the concentration of which an antibiotic reduces bacterial density by 1000-fold within 24 hours. Nonetheless, it should be noted that MIC and MBC measurements are highly affected by factors such as bacteria type and bacterial load, which means that bacteriostatic antibiotics can exhibit bactericidal activity and vice versa (Calhoun, Wermuth, and Hall 2021). There are several mechanisms in which antibiotics weaken or kills bacteria.

1.5 The Effect of Antibiotics on Cell Replication

Bacteria replication involves several proteins, which could serve as potential targets for antimicrobial compounds, yet antibiotics are primarily limited to inhibitors of DNA replication and translation (Eijk et al. 2017).

1.5.1 Inhibiting the DNA Replication

Most bacteria induce replication at a single site (e.g., in E. coli known as oriC). This site is recognized by an initiator protein known as DNA-A, which facilitates unwinding and therefore separation of the DNA-strands. Upon separation, replicative helicase (DNA-B) and DNA primase are loaded to form primosome complexes. Subsequently, DNA-B unwinds parental DNA strands, and DNA primase synthesizes short RNA primers. RNA synthesis leads to the assembly of DNA polymerase III holoenzyme (DNA III HE), which is the primary replication enzyme in bacteria. Nonetheless, the topology of DNA needs to be altered for DNA replication to occur. This is ensured by type II topoisomerases, including DNA gyrase and topoisomerase IV. DNA gyrase regulates supercoiling by inducing negative supercoils into relaxed DNA, whereas topoisomerase IV relaxes the negative supercoiled DNA (Robinson and Van Oijen 2013; Kapoor, Saigal, and Elongavan 2017). Inhibiting DNA replication involves topoisomerase II inhibitors, which target the DNA gyrase and topoisomerase IV. The inhibition of these enzymes interrupts the replication process of DNA, leading to DNA damage (Kapoor, Saigal, and Elongavan 2017; Eijk et al. 2017).

1.5.2 Inhibiting DNA Translation

The translational phase mainly revolves around tRNA, which has three main binding sites: the acceptor site (A-site), the peptidyl site (P-site), and the exit site (E-site). Upon initiation stage, the tRNA initiator, fMet-tRNA, binds to the P-site of the 30S ribosomal subunits. This binding leads to the recruitment of 50S subunits that result in the formation of a functional 70S ribosome, which then translates mRNA to proteins. (Robinson and Van Oijen 2013; Kapoor, Saigal, and Elongavan 2017). Inhibiting DNA translation involves disrupting the synthesis of proteins present in mRNA. This is achieved through the inhibition of the biosynthesis of 70S ribosome. Antimicrobials, therefore, target either the 30S or 50S subunit of the bacterial ribosome and alters its functionality (Kapoor, Saigal, and Elongavan 2017).

1.6 Targeting the Cell Wall with Antibiotics

Cell wall synthesis involves (1) cytoplasmic generation of the lipid-linked disaccharide-pentapeptide precursor lipid II, (2) translocation of lipid II to the outside of the cell by flippases and (3) the assembly of the cell wall by penicillin-binding proteins (PBPs) and Shape, Elongation, Division, and Sporulation (SEDS) proteins (Dörr, Moynihan, and Mayer 2019). This process is highly affected by undecaprenol, which is synthesized by the isoprenyl transferase enzyme UppS. Antibiotics target this enzyme, resulting in decreased synthesis of undecaprenol pyrophosphate, which disrupts the translocation of peptidoglycan precursor lipid II and thereby the cell wall synthesis (Dörr, Moynihan, and Mayer 2019).
In addition, cell wall synthesis can also be interrupted by β-lactam antibiotics. The target of these agents are PBPs. The agents work as an antagonist by mimicking the D-alanyl D-alanine portion of the peptide chain, which normally binds to PBP. The interaction between the β-lactam ring and PBP inhibits the synthesis of new peptidoglycan, which eventually leads to lysis of the bacterium (Kapoor, Saigal and Elongavan, 2017).

1.7 Multiresistant Bacteria - An Antibiotic Crisis

The discovery of antibiotics led to the belief that infections could be controlled and prevented. However, bacteria have now developed defence mechanisms against most antibiotics. These defence mechanisms have given rise to ARB, which are a result of overuse and misuse of antibiotics seen in clinical practice as well as in other industries such as livestock farming (Lee Ventola 2015; Kapoor, Saigal, and Elongavan 2017; Calhoun, Wermuth, and Hall 2021). Bacterial resistance can develop in the bacteria in several ways:

1. **Efflux pumps:** Membrane proteins that maintain low intracellular concentrations of antibiotics by exporting them outside the cell. This mechanism is performed at the same pace as when antibiotics enter the cell. The low intracellular concentration leads to decreased antimicrobials, which can reach the target of interest (C Reygaert 2018).

2. **Modification of target molecule:** Antibiotics are specific to the target molecule and therefore small variation in the target site of antimicrobials can exert a mechanism of resistance, since it prevents the drug from binding (C Reygaert 2018).

3. **Drug inactivation:** The bacteria can inactivate drugs either by degradation or by the transfer of a chemical group to the drug (C Reygaert 2018).

4. **Limiting drug uptake:** Porins within the outer membrane allows entrance of hydrophilic molecules. Antimicrobial agents use these porins to enter bacteria with a large OM. However, the bacteria can decrease the number of porins or through mutation change the selectivity of porins channel and thereby increase their resistance to antibiotics (C Reygaert 2018).

5. **Bacterial conjugation:** A process in which bacteria exchange or transfer genetic materials through cell-to-cell contact.

For on overview of antibiotic targets and resistance mechanisms, refer to Figure 2

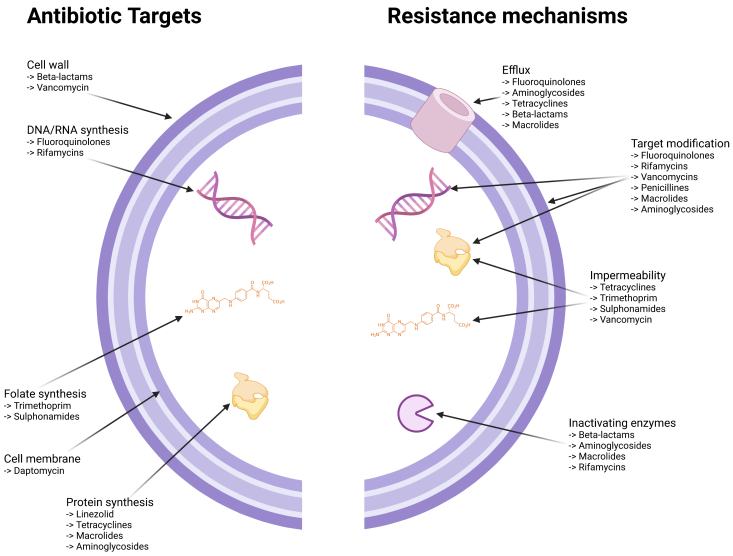


Figure 2: *An illustration of antibiotic targets and the bacterial resistance mechanisms - Created using Biorender and inspired by (Zhivich 2017)*

1.8 The Emergence of Resistant Pathogenic Organisms and Extended-spectrum-β-lactamases

Multidrug-resistant bacteria such as, *K. pneumoniae, S.pneumoniae, E. coli* and, *S. aureus* are, due to their high resistant profile, among the most critical bacteria (Shaikh et al. 2015). Furthermore, Enterobacteriaceae (Klebsiella and Escherichia) are believed to be the main source of ESBL, which are transmissible enzymes with the capability of inactivating penicillins, cephalosporins, and oxyimino-β-lactams through break down (Gupta et al. 2003; C Reygaert 2018; Shaikh et al. 2015). There exist several types of ESBLs and the most common genetic variant is currently CTX-M. However, genes such as SHV, TEM and, GES remains of high interest as SHV and GES are derived or described in *K. pneumoniae* and TEM was first reported in *E. coli* (Shaikh et al. 2015).

1.8.1 The different type of Extended-Spectrum-β-lactamase

CTX-M is a new arisen β-lactamase with the capability of hydrolyzing and inactivating cefotaxime and has most likely derived through horizontal gene transfer like plasmid conjugation, and it possesses highly potent hydrolytic activity against cefotaxime and other β-lactams (Shaikh et al. 2015).

SHV and TEM SHV and TEM have similar inhibition profiles, as both confer resistance to broad-spectrum penicillins. However, TEM is also capable of hydrolysing first generation cephalosporins. SHV and TEM are, unlike CTX-M, derived through amino acid substitutions (Shaikh et al. 2015).

GES GES-1 is another ESBL, which was described in *K. pneumoniae* and this gene provides the bacteria with defence mechanisms against penicillins and extended-spectrum cephalosporins (Shaikh et al. 2015).

With the broad resistance which the plasmid-mediated ESBL possess, it is necessary for clinical laboratories to rapidly detect their occurrence. This is to ensure, that the correct

therapy is initiated as early as possible. The current treatment for ESBL includes carbapenems or use of mechanism-based inhibitors, such as tazobactam, sulbactam, and clavulanic acid. Many studies have adressed ESBL-producing infections with *K. pneumonia*, which is known to adept well to the hospital environment (Kalp, Bethel, and Carey 2009; Paterson and Bonomo 2005).

The increased level of multidrug-resistant bacteria has resulted in the need for new antimicrobial agents for controlling and treating infections. However, with the bacteria's high adaptability and evolutionary capabilities, other interventions, like improving AST, must also be considered (Szymańska et al. 2018).

1.9 The Antibacterial Potential of Phytochemicals

The emergence of multidrug-resistant bacteria has drawn attention towards the study of plants and their antibacterial activity. The interest in using plants for treatment of various diseases has increased globally as approximately, 25 % of most prescribed drugs are obtained from natural sources, but around 350.000 plant species have been identified and the pharmacological potential of most of them are yet to be understood (Wintola and Afolayan 2015; Barbieri et al. 2017). Phenolic compounds play a pivotal role in both human and animal diets, which is a result of their antioxidant properties. Furthermore, phenolics have also exhibited other important abilities such as antimicrobial, which makes them interesting sources for antibiotics. Halophytes are plants that thrive in high levels of salinity and can withstand severe environmental conditions. These adaptive responses are associated with the synthesis and accumulation of various protective compounds, including phenolics. *S. herbacea*, as an example, exhibited high phenolic richness and an antimicrobial activity. Furthermore, the results from Essaidi et al. indicated that the antimicrobial activity was higher in the gram-positive as compared to gram-negative. The antibacterial properties have mainly been associated with phytochemicals, which are secondary metabolites designed to defend organisms against viruses, bacteria, and fungi. This is one of the reasons that herbal remedies have become a major topic in microbiology (Barbieri et al. 2017; Lopes et al. 2021). Despite these findings, the antimicrobial properties of halophytes have not been extensively studied. It is therefore of great interest to analyse these properties as it offers benefits to society. The antibacterial potential demonstrated in *S. Herbacae*, is, potentially, related to their content of phenolic compounds such as phenolic acid and flavonoids (Essaidi et al. 2013; Lopes et al. 2021).

1.9.1 The Antimicrobial Effect of Phenolic Acid

Phenolic acids are secondary metabolites from plants that kill microorganisms and/or inhibit the growth of bacteria as part of the antimicrobial mechanisms. The metabolite exerts its antibacterial effect through numerous mechanisms, which include (1) destabilizing the cytoplasmic membrane within the bacteria, (2) altering the permeability of the plasma membrane, (3) inhibiting extracellular microbial enzymes, (4) directly altering microbial metabolism, and (5) depriving microbes of substrates required for growth. These effects and how they are exerted depends on the molecular structure of the phenolic acid (Liu et al. 2020).

1.9.2 The Antimicrobial Effect of Flavonoids

Flavonoids are polyphenols and so far, 8000 subclasses have been identified and divided into the following groups: flavonols, flavones, flavanols, flavanones, anthocyanidins, and isoflavonoids. Among those, flavanols and flavonols have demonstrated the highest antibacterial activity through several mechanisms, including (1) inhibition of bacterial virulence factors (enzymes and toxins), (2) interaction with cytoplasmic membrane, (3) suppression of biofilm formation, and (4) by exerting a synergistic effect with antibiotics. Their mechanism of action depends on the type of flavanol and flavonol (Miklasińska-Majdanik et al. 2018).

1.10 Technology for Antibiotics Susceptibility Testing and Biotyping

Multidrug-resistant bacteria have resulted in limited treatment options. This is of great concern as current AST is time-consuming and resulting in delayed initiation of treatment. This is troublesome as the best clinical outcome relies on optimal selection, dosing and duration of antimicrobial treatment (J, F, and Cannady 2021). New rapid diagnostic methods for faster identification of resistance mechanisms are therefore needed to ensure faster treatment with the right antibiotic. Currently, no method is used, which identify a bacterium and antibiotic susceptibility (Blumenscheit et al. 2020). Currently, Matrix-Assisted Laser-Desorption/Ionisation – Time of Flight Mass Spectrometry (MALDI-TOF MS) has become an accepted standard approach for bacterial identification as it, compared to biochemical tests, provides faster and more accurate results, while also being cheaper for large laboratories. In addition, the golden standard for AST analysis, is a disk diffusion method or a dilution method, both of which involves growth of bacteria in the presence of antibiotics (Charretier et al. 2015). However, the preferred method when analysing antibacterial activity of plants and extracts is the agar well diffusion method (Balouiri, Sadiki, and Ibnsouda 2016).

1.10.1 Disk Diffusion Method - The Golden AST Standard

The disk diffusion method is performed using an isolated bacterial colony suspended into a growth medium and then standardized through a turbidity test. The standardized suspension is subsequently inoculated onto a solidified agar plate, with an antibiotic coated paper tapped on it. The presence of an antibacterial effect forms an inhibition zone, which is measured and compared to a commercial antimicrobial susceptibility platform, for the respective antibiotic (Khan, Siddiqui, and Park 2019). This method is a simple and cost-effective approach to assess bacterial susceptibility, but lacks time-efficiency. Ideally, one would prefer that susceptibility data are available as early as possible as empiric treatment is initiated during the process (Khan, Siddiqui, and Park 2019; Rentschler, Kaiser, and Deigner 2021).

1.10.2 Agar Well Diffusion Method

As mentioned, the agar well diffusion method is a widely used approach to analyse the antimicrobial activity of plants and microbial extracts. The method is similar to the disk diffusion method, as it involves the inoculation of bacteria onto an agar plate. However, with this method, a 6-8mm hole is punched and a volume of 20-100µL antimicrobial agent is added to the well. The plate is left under suitable conditions and the extract diffuses into the agar medium and if an antimicrobial effect occurs a zone of inhibition is present (Balouiri, Sadiki, and Ibnsouda 2016).

1.10.3 Optical density test

Despite the disk and agar well diffusion method being the most appropriate for testing bactericidal activity, the optical density test remains more appropriate when analysing the bacteriostatic effect, as it illustrates bacterial growth over a time-period and gives a time-dependent or concentration-dependent antimicrobial effect (Balouiri, Sadiki, and Ibnsouda 2016).

1.10.4 MALDI-TOF Biotyping

With the emergence of MALDI-TOF MS in the clinical setting, identification of bacteria has increased both in relation to sensitivity and accuracy. This has also been revealed by studies, which demonstrated that MALDI-TOF MS is able to discriminate methicillin-resistant *S.*

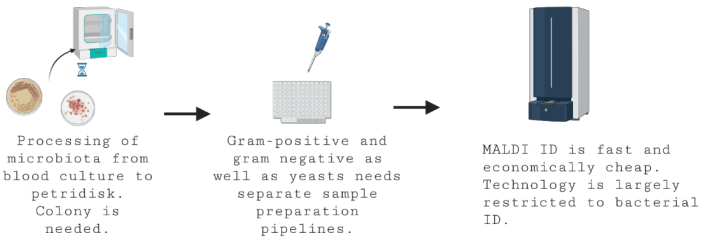


Figure 3: *An overview of the MALDI-TOF MS workflow - Created using BioRender*

aureus from methicillin-sensitive *S. aureus*. Bacteria with the MALDI-TOF MS are identified based on their unique bacterial mass spectrum, with findings generated on-site to a database with spectra obtained from pure bacterial colonies. The advantages MALDI-TOF MS possesses has led to several studies proposing multiple approaches for AST using MALDI-TOF MS. However, according to Rentscler et al., the MALDI-TOF MS lacks efficiency in relation to assessing polymicrobial samples, which the LC-MS/MS offers (Welker and Belkum 2019; Dimard E. Foudraine et al. 2019; Khan, Siddiqui, and Park 2019; Rentschler, Kaiser, and Deigner 2021). An overview of the MALDI-TOF MS process is depicted in Figure 3

1.10.5 LC-MS/MS as the Future AST and Bacteria Identification Tool

Despite MALDI-TOF MS's potential in AST, LC-MS/MS provides faster and more sensitive results. Empiric treatment of infections are initiated and later adjusted according to antibiotic susceptibility data, and it is, therefore, ideal to improve pathogen identification and AST to ensure a faster turnaround time and initiation of correct therapy, which LC-MS/MS has the potential to. LC-MS/MS allows detection of several peptides, which makes it possible to associate specific peptides as well as their quantity with specific resistance mechanisms (Charretier et al. 2015; Suhandynata et al. 2021). An overview of the LC-MS/MS workflow is illustrated in Figure 4

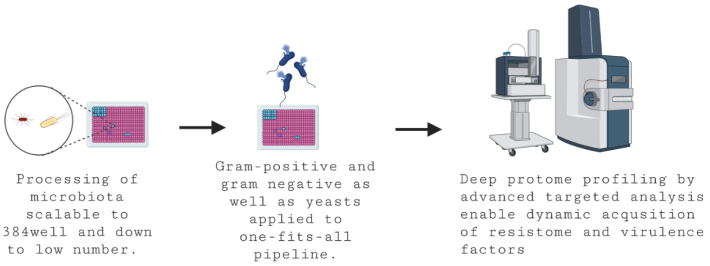


Figure 4: *An overview of the LC-MS/MS workflow - Created using BioRender*

A study by Suhandynata et al., demonstrated that the β-lactamase activity can be measured with LC-MS/MS by analysing the hydrolysis of antibiotics, which existing studies have shown (Suhandynata et al. 2021).

1.10.6 The Liquid Chromatography Mass Spectrometry System

The mass spectrometry is an analytical system, which possess the capability of analysing biomolecules including proteins. It has always been desireable to couple the LC to MS as this would increase sensitivity and specificity. This is achieved through a separation phase using

the chromatography and an electrospray ionisation technique. Here, the proteins are converted into charged droplets, which the mass spectrometer detects based on the mass-to-charge ratio, which is then presented in a spectrum (Aitken 1998; Pitt 2008). All mass spectrometers are loaded with a vacuum, which ensures free movement of the ions. This is to make sure that ions do not interact and exchange energy, which would affect the mass-to-charge ratio (Aitken 1998). With the electrospray ionisation technique, the analyte is sprayed into an electric field, which creates small, charged droplets that can be described as a mist. The droplets evaporates in the high-vacuum region until a single species of charged analyte is reached (Aitken 1998).

The sample is subsequently transferred to a mass analyzer, there exists several types, the quadrupole is mainly the one which is used in combination with Lc-MS/MS. The quadrupole invovles four parallel placed cylindrical rods, which creates a varying electrical field that separates and releases ions based on the mass-to-charge ratio (Aitken 1998).

Data-depended and independent acquisition: are, currently, the acquisition modes detached to acquire MS and MS/MS spectra for all the components in a single analytical run. In general the Data-depended acquisition mode is performed to assess the most abundant proteins, whereas the independent acquisition mode all precursor ions are isolated. (Sun et al. 2020).

2 Aim and Strategy

2.1 Aim

With fewer antibiotics available to treat infectious diseases, more lives are at risk. The antibiotic stewardship program aims at optimizing antibiotic treatment by preventing the misuse and overuse of antibiotics in the clinical setting. This will help prevent the expansion of multidrug-resistant bacteria and ensure optimal treatment outcome. However, with the current AST method, this remains troublesome. It is, therefore, necessary to initiate effective antibacterial treatment, as this has shown to have a better outcome for the patient, which rely on quick identification of resistance (J, F, and Cannady 2021). For this reason, new antibiotics and faster AST methods, which can accurately identify resistance mechanisms, are needed. In this study, we aim at (1) investigate the antibacterial activity of phytochemicals and (2) analyse the potential of using LC-MS/MS for bacterial resistance identification, using bacteria strains identified with MALDI-TOF MS. An overview of the workflow for both aims is depicted in Figure 5.

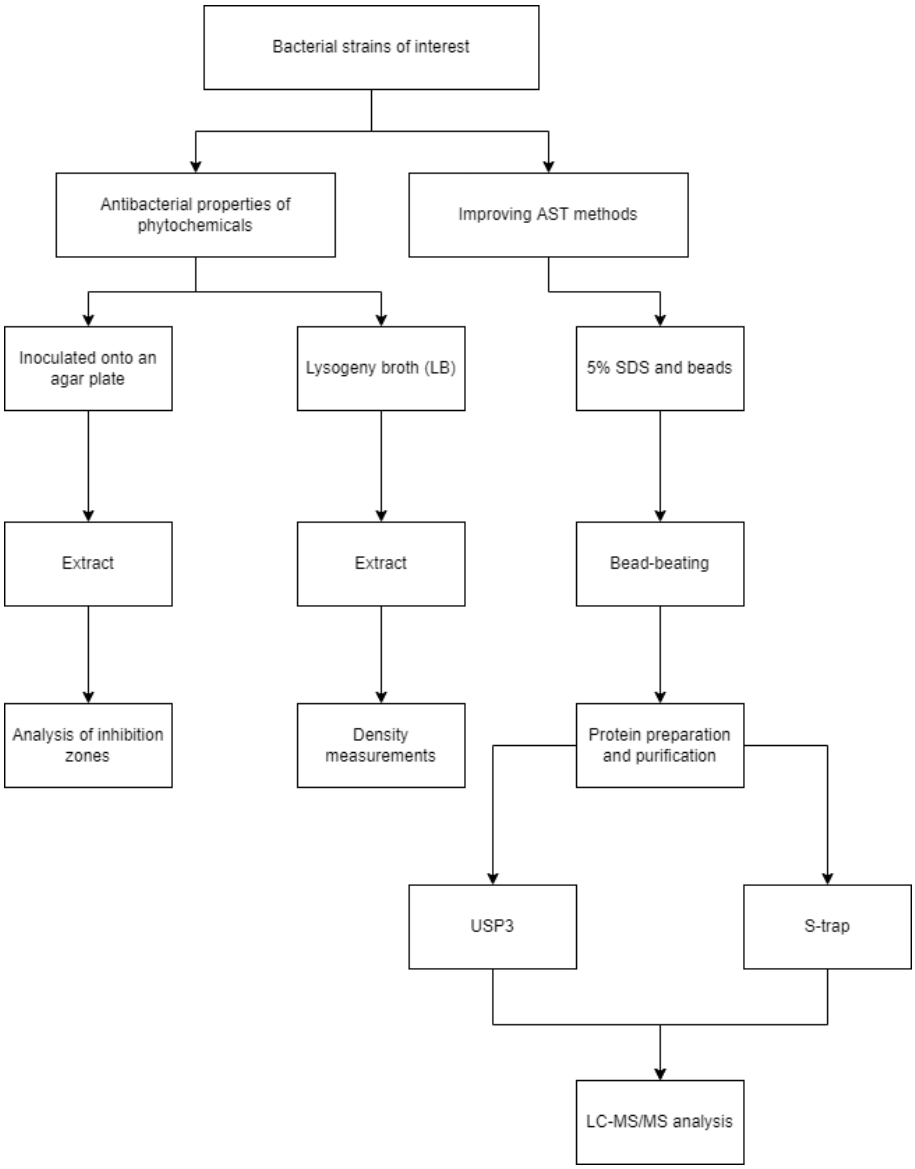


Figure 5: *An overview of the workflow in this study*

2.2 Strategy

2.2.1 Testing the antibacterial properties of phytochemicals

For this project, phytochemicals, extracted from *S.ramosissima*, are analysed for their antibacterial effect through two methods. (1) With the focus on assessing the bactericidal effect and (2) estimating the bacteriostatic effect. Bactericidal analysis will be conducted by performing an agar well diffusion test. In this test, the bacteria will be inoculated unto an agar plate loaded with the extract. The bacteria are then left under suitable conditions prior to analysing and measuring inhibition zones. Furthermore, an optical density test will be performed to estimate the bacteriostatic effect of the phytochemicals. Here, the bacteria are transferred to a suspension consisting of lysogeny broth (LB) and extract. Density measurements will then be taken to estimate the bacteria's growth capability in the extract.
A test sample will be run on a completely sensitive *S. aureus*, this is done in order to be certain that there is an antibacterial effect. Subsequently, the test will include *K. pneumoniae*, *S. pneumonia*, and *E. coli* strains.
A patent secured extract will also be tested on gram-positive bacteria only, as no content information was provided.

2.2.2 Assessing Mass Spectrometry potential for AST

In this study, we aim at, using LC-MS/MS, analysing the potential of determining antibiotic resistance based on bacterial proteins as compared to enzymatic activity (Suhandynata et al. 2021). To investigate this, bacterial proteins will first need to be extracted, which relies on disruption of the cell wall. Bead-beating has demonstrated the highest yield in relation to protein extraction and will therefore be the preferred method in this study. Subsequently, proteins need to be cleaved and purified before MS-analysis is conducted. With the interest of having a fast identification, in this study, two methods were tested. The USP3 method, which has shown to possess the highest yield, and the S-trap, which is faster in relation to preparation (Hayoun et al. 2019). The objective was to analyse, using LC-MS/MS, the highest protein ID's and compare it to the time needed for sample preparation. With Enterobacteriaceae producing ESBL we chose to use a *K. pneumonia* strain (ATCC343), as a total of three *K. pneumonia* strains will be included in this study. The chosen method will then be performed on all three strains to compare the degree of resistance in each.

3 Methodology

In this study, several methods and protocols were performed. However, Figure 6 gives a quick overview and insight on the setup of this project.

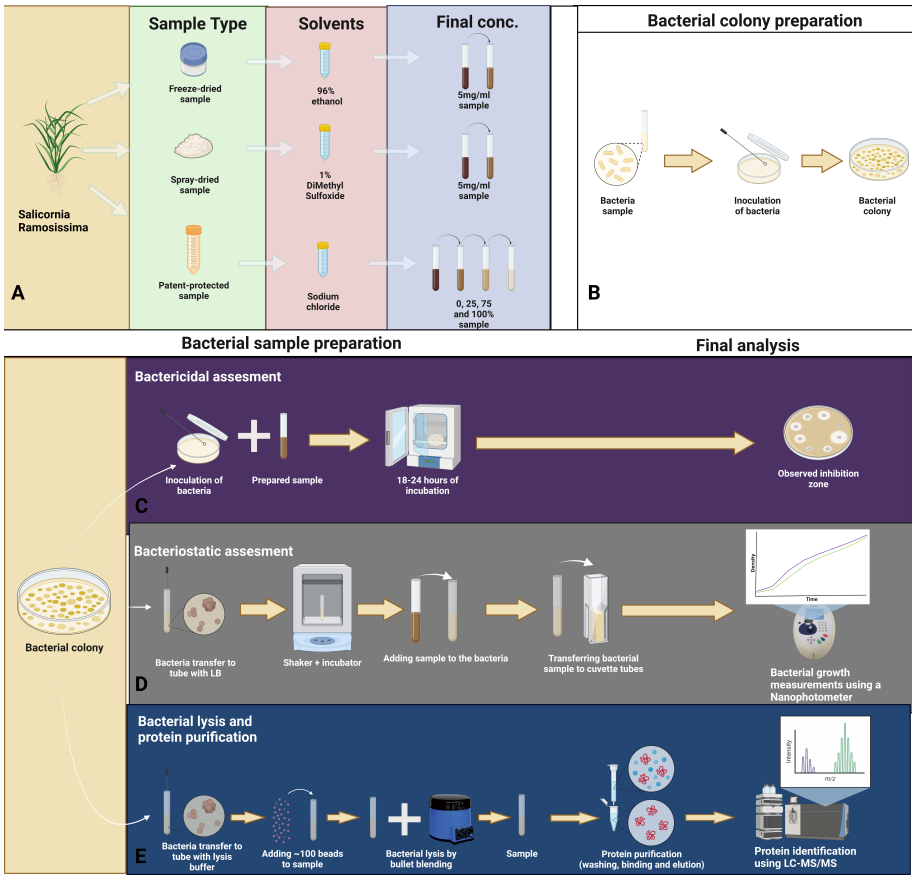


Figure 6: *An overview of the preparation and analytical methods performed. (A) The three sample types received were diluted in a respective solvent to the concentration of interest. (B) A bacteria suspension was inoculated onto its respective plate and left to grow. (C) A bacterial colony, from the prepared plate, was inoculated onto a new plant with the presence of the prepared samples. The plate was then left in the incubator and inhibition zones were subsequently measured. (D) A bacterial colony was transferred to a tube with LB and left to grow in the incubator. Subsequently, sample was added to the tube and density measurements were performed over a time-period. (E) A bacterial colony was transferred to a tube with lysis buffer and beads and bullet blended, to disrupt the bacteria cell wall. Released bacterial proteins were then prepared and purified before loading them onto LC-MS/MS - Created with Biorender.*

3.1 Bacterial Strains

The bacterial strains used in this project include three *K. pneumoniae* (ACC116180, ACC117196, ACC343), which presents three different type of resistance coverage. Furthermore, one *E. coli*, one antibiotic sensitive *S. aureus* and, one penicillin-resistant *S. pneumonia* were also included. All strains were used for the assessment of antibacterial capabilities of phytochemicals, but only the Klebsiella strains were used for MS-analysis.

3.2 Bacterial Preparation

Bacteria were inoculated and incubated at 37° for 18-24 hours. A bacterial colony was, subsequently, transferred to a new plate for pure cultivation. Prior to bactericidal analysis, the bacterial concentration was adjusted according to a McFarland of 0.5. This was done by adding a bacterial colony in a salt buffer and measuring the density with a McFarland densitometer. Prior to bacteriostatic assessment, the bacteria were left to grow in 4 ml LB for 18-24 hours in a vortex incubator. Bacterial MS analysis involved adding 4 baterial colonies to a Rino Microcentrifuge tube with 100µL of beads and 200µL of lysis buffer. The sample was subsequently, boiled for 5 minutes at 95° and then Bullet Blended for 5 minutes at level 8 followed by a centrifugation for 5 minutes at 14,000 x g at 4°.

3.3 Preparation of Bioactive Extract

The aqueous or ethyl acetate soluble fractions of Salicornia sp. were prepared by soxlet based extraction of secondary metabolites from lignified plant tissue. Approximately 0.5g of freeze-dried (FD) extract and 15g of spray-dried (SD) extract were received from AquaCombine consortia at AAU Esbjerg. Furthermore, 50ml of three patent protected (PP) samples were also received from AAU Esbjerg where the extraction process are protected due to patent claims. The FD sample was diluted to 50mg/ml in ethanol and subsequently diluted in LB in a 1:10 ratio. This was done to prevent a bactericidal effect from ethanol. The SD sample was diluted to a concentration of 5mg/ml in 1% DMSO. A control was made for both sample types, with ethanol, diluted in LB at the same ratio, acting as a control for the FD and 1% DMSO for the SD. The PP samples were diluted in NaCl and tested for antibacterial properties at a concentration of 25, 75 and, 100%, where pure NaCl acted as a control.

3.4 Agar Well Diffusion - Assessing the Bactericidal Properties of Phytochemicals

Bacteria were inoculated on an agar plate and wells were subsequently made by using an 8mm puncher. Next, 100µL of sample was added, and the plate was incubated for 18-24 hours at 37°. Inhibition zones were then measured.

3.5 Optical Density Test - Assessment of the Bacteriostatic Effects of Phytochemicals

For bacteriostatic assessment, FD, SD, and 100 % PP extracts were used. The sample consisted of 250µL of bacteria diluted in 1000µL extract and 8.75mL LB and a dilution with distilled water substituting the extract acted as a control. The optical density, representing bacterial growth, was measured every hour on an Implen Nanophotometer at a wavelength of 600nm. To ensure the best possible readings, blanks were prepared and loaded between each measurement. Blanks consisted of the same mixture as the sample, but free of bacteria.

3.6 Protein Extraction using USP3

The lysed bacteria were added with a 1:10 ratio of prepared PureCube Carboxy Magbeads and 50µL of 100% acetonitrile. The sample was then incubated for 15 min at 400RPM using a table-top thermomixer shake and subsequently incubated for 2 min on a magnetic rack to immobilize beads. The supernatant was removed and the beads were washed twice with 200µL of 80% ethanol and once with 180µL of 100% acetonitrile, vortex was done in between each

wash. A digest solution, with trypsin, was then added in a 1:20 ratio and incubated for 60 minutes at 600RMP at 37°The sample was then acidified in 5µL of 5% TFA and vortexed. Finally, the beads were immobilized and peptides were transferred to a new tube.

3.7 Protein Extraction using S-trap

50µL of lysed bacteria was transferred to a 0.5mL Eppendorf Lobind tube and phosphoric acid was added until a pH of >1 was achieved. The sample mixed with phosphoric acid was subsequently vortexed, mixed with 350µL binding/wash buffer, and spinned briefly. The whole sample was then transferred to an S-trap mini and centrifuged for 30 seconds at 4,000 x g at 4°. The tube was then washed with 400 µL binding/wash buffer and centrifuged at the same settings. This step was repeated three times and flow through was removed in between. The spin column of the S-trap mini was subsequently transferred to a 1.5mL LoBind Eppendorf tube and added with 125µL digestion buffer containing trypsin in a (1:10 wt/wt). The sample was next incubated in a water bath for 5 minutes at 50° and subsequently mixed with 80µL of elution buffer and centrifuged for 30 seconds at 4,000 x g at 4°. This step was repeated for each of the three elution buffers used (1) containing 50mM TEAB in water, (2) containing 0.2% FA in water and, (3) 50% ACN in water.

3.8 Rapid Tandem MS based Biotyping

Analysis of bacterial proteins was performed with the use of Evosep One LC system coupled to a trapped ion mobility spectrometry (TIMS) quadrupole time-of-flight (TOF) MS/MS. The system was supported with a parallel-acquisition series fragmentation (PASEF) (Bruker, Bremen, DE) technique, analysed in a data-dependent acquisition (DDA) mode.

The peptides from tryptic digestion were desalted using Evotip based desalting according to manufactures recommendations. The mobile phases of this system consisted of mobile phase A and B. Mobile phase A comprised 0.1% FA in MS grade water, whereas mobile phase B comprised 0.1% FA in ACN. The analysis was conducted with a fixed sample throughput of 30 to 200 samples per day with optimized LC duty cycle. Protein identifications were subsequently searched in Biognosys SpectroMine (v. 3.2.220222.52329) against a ESBL optimized database, which contained annota- tions for ESBL and other antibiotic resistance mechanisms. The Comprehensive Antibiotic Resistance Database (CARD) database (https://card.mcmaster.ca/) was used for protein identification based on antibiotics resistance associated gene products only.

3.9 Statistical Analysis

Statistical analysis was performed using Rstudio (v.RStudio 2022.02.1+461 "Prairie Trillium").

4 Results

The bactericidal effect of phytochemicals were assessed by measuring the zone of inhibition, whereas the bacteriostatic capability was determined through density measurement over the course of 6 hours, with measurements taking place every hour.

4.1 The Bactericidal Effect of the Phytochemicals in Salicornia

A antibiotic sensitive wildtype *S. aureus* was inoculated on an agar plate and subsequently FD and SD samples, diluted in 1% DMSO or ethanol and LB, were loaded onto the agar well at a concentration of 5mg/ml. The bacterium was subsequently left to grow before analysing the presence of an antibacterial effect.

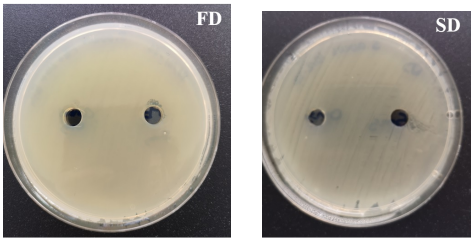


Figure 7: *Results depicting the zone of inhibition for the FD and SD samples at a concentration of 5mg/ml extract*

Based on the Figure 7, no zone of inhibition was observed, and it can therefore be elucidated that the samples did not have any antibacterial effect did not on a completely sensitive *S. aureus* strain.

4.2 The Bactericidal Effect of Patent-Protected Samples

A completely sensitive *S. aureus* and a *S. pneumonia* were inoculated onto an agar plate, with the presence of PP sample at a concentration of 25, 75, and 100%. The bacteria were left to grow prior to analysing the antibacterial effect.

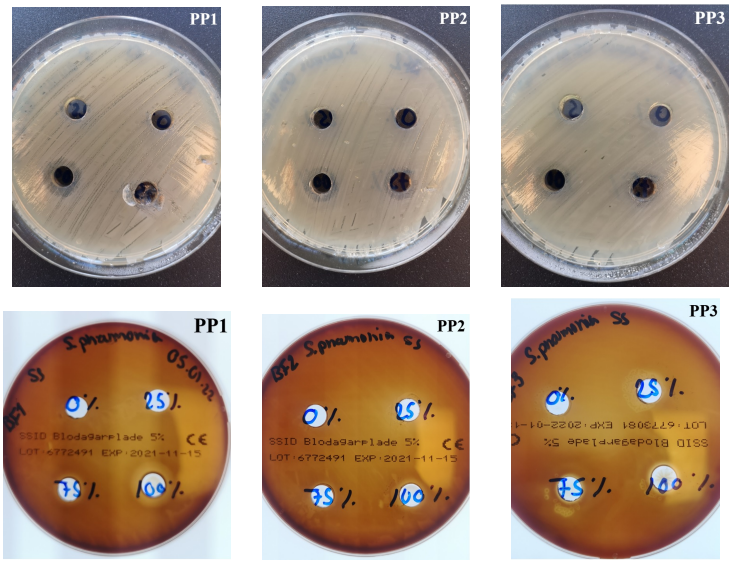


Figure 8: *An overview of the antibacterial capabilities of the patent-protected (PP) samples on gram-positive bacteria*

From Figure 8 it can be elucidated that the patent-protected samples demonstrated an antibacterial effect at a concentration of 75 and 100% on both *S. aureus* and *S. pneumonia*. However, no inhibition zone was observed in *S. pneumonia* loaded with PP3.

4.2.1 The Bacteriostatic Effect of the Prepared Extracts

A completely sensitive *S. aureus* strain was mixed with extract and LB. Density measurements were then performed every hour to illustrate the bacterial growth over time. Based on the graph, the PP1 and PP3 extracts demonstrated a bacteriostatic activity and statistical analysis also supports this as a significant difference was found, which is illustrated in Table 2.

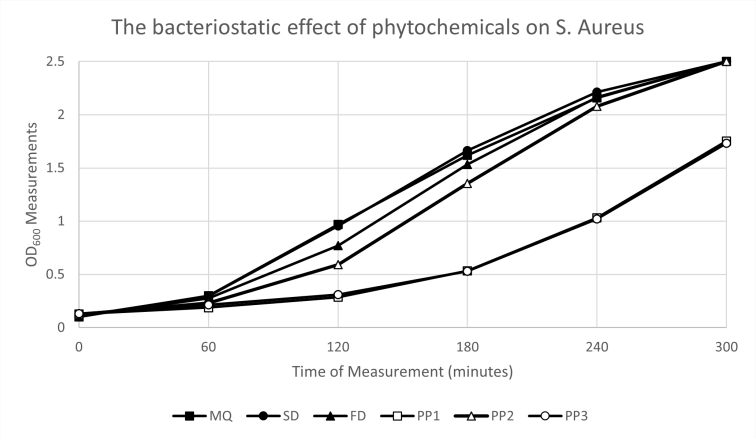


Figure 9: *An illustration of the bacteriostatic effect of all compounds tested. The graph depicts the density over time with a one-hour interval*

By figure 9, it can be elucidated that the PP's possessed bacteriostatic potential, which was also expected due to their bactericidal results.

Extract	Extract	P-value
MQ	FD	0.9992575
PP1	FD	**0.0074124**
PP2	FD	0.9941712
PP3	FD	**0.0078123**
SD	FD	0.9976640
PP1	MQ	**0.0031634**
PP2	MQ	0.9477301
PP3	MQ	**0.0033380**
SD	MQ	0.9999989
PP2	PP1	**0.0262355**
PP3	PP1	1.0000000
SD	PP1	**0.0025071**
PP3	PP2	**0.0275654**
SD	PP2	0.9219584
SD	PP3	**0.0026461**

Table 2: *An overview of the p-values estimated by Tukey's Test*

4.3 Resistance Proteins Identified with the use of MS

4.3.1 Comparison of USP3 and S-trap

In this project, we analysed and compared the time needed to extract resistance proteins and identify them using LC-MS/MS. Prior to MS-analysis, the proteins were cleaved using either the USP3 or S-trap method. Based on the MS-analysis, we managed to identify 15 and 16 protein groups using S-trap and USP3, respectively. However, taking the time needed into consideration, the S-trap yields a better result and was therefore used on the included strains.

4.3.2 Identified Proteins using the S-trap method

The bacterial cell wall was disrupted, to allow protein availability, using bead-beating. The proteins were then cleaved to peptides, which were loaded into the LC-MS/MS. The results from the LC-MS/MS scans are depicted in Table 3.

Protein	ATCC116180	ATCC119671	ATCC343
CRP	175854	112818	76362
LptD	67648	18309	49507
Ugd	47702	8881	23950
rpoB2	48342	18991	9165
OMpK37	172709	367412	108936
OmpA	1023653	1358664	893167
AAC(6')-30/AAC(6')Ib	63307	57218	39861
APH(3')-Ia	44981	N/A	22877
APH(3")	12450	N/A	N/A
AAC(6')-Ib-cr6	5928	34334	N/A
AcrA-KPN	141401	46384	106990
AcrB	13016	1528	4812
Acra-e.c	67648	18309	49507
TolC	77420	31725	34964
MexE	31165	15608	33362
H-NS	223995	53447	266806
SHV-65	30436	N/A	50310
Sul2	51000	6615	N/A
pp-cat	483352	N/A	N/A
TEM-55	19662	N/A	N/A
ANT(2")	122755	N/A	N/A
msbA	780	N/A	N/A
QnrB1	N/A	6666	N/A
CTX-M-82	N/A	22667	N/A
KPC-7	N/A	N/A	171600
oqxA	N/A	N/A	16413
oqxB	N/A	N/A	13923
emrR	N/A	N/A	705
OHIO-1	N/A	N/A	2772
mphA	N/A	N/A	569

Table 3: *An overview of the proteins found and their quantity in the different strains tested*

The potential use of LC-MS/MS for the identification of resistance was assessed and demonstrated promising results, in total 30 proteins were found with 17 of these being unique (only found in one strain). The strain with the highest protein count was ATCC116180, with a total number of 22 proteins. 21 was found in the ATCC343 strain, and only 17 was found in ATCC119671. An overview of the proteins and quantity within the different strains is illustrated in Table 3.

5 Discussion

With multidrug-resistant bacteria challenging the health care with limited treatment options and with the time-consuming AST, the future of infectious treatment becomes dire. The "post-antibiotic" era is a serious threat, which needs attention. We, therefore, focused on improving these aspects of infections treatment by assessing the potential of new antibiotics and methods for AST (O'Neill 2016; *Challenges to Tackling Antimicrobial Resistance* 2019; *Landscape of diagnostics against antibacterial resistance, gaps and priorities* 2019).

5.1 The Potential use of Phytochemicals as Antimicrobial Drugs

In this study, we assessed the antibacterial properties of phytochemicals using extracts from *S. ramosissima*. The need for new or supplementing antibiotics is crucial as the occurrence of multidrug-resistant bacteria is expanding and opposing a serious threat to the public health care, since bacteria are constantly altering the bactericidal effects of new antibiotics, through several resistance mechanisms, limiting treatment options (*Challenges to Tackling Antimicrobial Resistance* 2019). To the best of our knowledge, this is the first study analysing antibacterial properties using *S. ramosissima*. However, existing research has analysed other plants related to the specie, mainly *S. herbacae*, which demonstrated antibacterial properties on both gram-positive and gram-negative bacteria by Essaidi et al. and Rad et al. (Essaidi et al. 2013; J. S. Rad, Alfatemi, and M. S. Rad 2014). The antibacterial effect have been linked to phytochemicals, such as phenolic compounds. However, studies have shown that components such as fatty acids and osmotic compounds could contribute to the antibacterial properties, suggesting a synergetic effect (Essaidi et al. 2013).

This could prove to be the missing piece in this study, since the level of other components has not been assessed. For future studies, it is therefore advised that the synergistic effect of phytochemicals with several compounds is considered. However, antibacterial properties, against gram-positive bacteria, were observed in the PP samples, with PP1 and PP3 showing a statistical significant difference compared to the other extracts. An overview of the different p-values and comparison between extracts is depicted in Table 2 and Figure 9. Nonetheless, due to patent on the PP samples, no information was provided, but based on the observed results, there is a potential antibacterial capability. However, it cannot be elucidated that the antibacterial effect is a result of phytochemicals only, as there might be other compounds acting synergistically.

Table 4: An overview of phenolic compounds and the concentration

Phenolic Compound	**conc.mg/g**
Acide Protocatechuique	0.685
Acide Coumarique	0.604
Acide Vanillique	4.830
Acide Caféique	1.775
Acide Férulique	7.768
Quercétine	0.186
Isorhamnétine	0.069
Acide Néochlorogénique	LQ
Acide Cryptochlorogénique	LQ
Acide Chlorogenique	LQ
Kaempferol-3-Glucoside	0.263
Hyperoside	0.926
Isoquercitrine	10.912

Nonetheless, it should be noted that a study by Barbieri et al. explains the complications of implementing phytochemicals into monotherapy, which is mainly due to the high MIC needed that could account for the lack of an antibacterial effect seen in this study. Barbieri et al. estimated that the MIC of phytochemicals should be ranging from 100µg/ml to 5000µg/ml. In this study, the highest concentration of phenolic compound was estimated to be 10.912 (mg/g). An overview of all the phenolic compound and concentration in the samples tested is depicted

in Table 4 (Barbieri et al. 2017).

5.2 The Potential of Identifying Resistance Proteins with MS

In this study, we analysed the potential of using LC-MS/MS for faster identification of bacterial resistance, with the focus on ESBL. By implementing LC-MS/MS in AST, identification of antibiotic resistance mechanism could be improved and result in faster initiation of antimicrobial therapy with the correct antibiotic. This is of great importance as studies have shown that the increase in drug-resistant infections could lead to many lives being at risk and that uncontrolled systemic infections could prove fatal, especially within the first 24-48 hours.

Our findings, suggests that LC-MS/MS could potentially prove useful in relation to this complication. Using this system we managed, within the same-day of analysis, to identify and quantify a total of 30 resistance proteins, three of which are associated with ESBL (TEM-55, SHV-65, and CTX-M-82). SHV and TEM mutations are increasing ESBL's hydrolysis of β-lactams, and SHV-65 was found in two of the three strains. Namely, in ATCC343 and ATCC116180, with a quantity of 50310 and 30436, respectively. Nonetheless, despite the lower quantity of SHV-65 in ATCC116180, this strain also possessed the TEM-55 mutation with a quantity of 19662. Lastly, we identified the CTX-M-82 in ATCC119671 with a quantity of 22667. What is interesting is that according to Shaikh et al. studies have shown higher efficacy of enzyme-inhibition with tazobactam as compared to sulbactam or clavulanic acid, which is crucial as this will determine the most beneficial combination therapy and thereby most efficient treatment option for this variant (Shaikh et al. 2015; Paterson and Bonomo 2005). Furthermore, improving AST to same-day results would also provide benefits in relation to both the clinical setting and infection control, which is the objective of the antibiotic stewardship program. Here, correct dosing of the antimicrobial agent is a keynote in treatment of pathogenic infections, which is troublesome, has the disk diffusion method does not provide a MIC-value (Suhandynata et al. 2021; J, F, and Cannady 2021; Rentschler, Kaiser, and Deigner 2021). Our results indicate that bacterial resistance proteins can be identified, within the same day of analysis, using LC-MS/MS and therefore the potential of assessing resistance profile of clinical isolates. This is further supported by a study conducted by Serafim et al. and Suhandynata et al., which assessed antimicrobial resistance evaluation based on antibiotic hydrolysis. Serafim et al., demonstrated a 100% specificity between LC-MS/MS scans and MIC-tests for several antibiotics. However, in this study, we identified resistance level based on proteomics as compared to hydrolysis.

5.2.1 Evaluation of methods for protein cleavage and purification

Additionally, to the objective of this project, we also evaluated time-efficiency of the USP3 and S-trap method. Our result demonstrate that using the USP3 resulted in one extra protein group compared to the S-trap. However, when analysing the time for completion, the USP3 takes approximately one and a half hour, which is a result of the 1 hour digestion step. This step only takes five minutes in the S-trap protocol, which in overall takes approximately 30 minutes to complete. This makes S-trap favorable, in relation to this study's aim, compared to the USP3.

6 Conclusion

6.1 Phytochemicals - Antibacterial but Hard to Implement

The occurrence of multidrug-resistant bacteria, such as ESBL-producing microorganism, has become a major health care problem. With bacteria exerting resistance to several drug classes, treatment options have become limited. Phytochemicals are compounds found in plants and are designed to defend organisms against viruses, bacteria, and fungi, which have led to the interest of analysing their potential use as antibiotics or as an antibiotic supplement. We assessed the antibacterial capabilities of phytochemicals extracted from *S. ramosissima* on a completely sensitive *S. aureus*. However, we did not observe any effect with an extract concentration of 5mg/ml, which might be a result of a low phytochemical concentration as studies have shown that the phytochemical concentration should range from 100µg/ml to 5000µg/ml, before an effect is observed.

6.2 LC-MS/MS - Promising Results Demonstrate AST Potential

In this study, we investigated the potential use of LC-MS/MS for AST. The need for a fast and optimal AST method is important as it results in faster initiation of treatment with the correct drug. Currently, the disk diffusion method is the golden standard for AST, but susceptibility results can take up to 72 hours to obtain. The patient is, until susceptibility data is available, treated empirically, which is not sufficient, as one of the major contributing factors is misuse and overuse of antibiotics. Susceptibility data therefore needs to be available faster to decrease the emergence of multidrug-resistant bacteria. Among these are ESBL-producing microorganisms which, due to their broad resistance profile, have become difficult to treat. This plasmid-mediated enzyme exerts resistance towards several β-lactams, but also other antibiotic classes, which limits treatment options. We extracted bacterial proteins from three *K. pneumoniae* strains (ATCC116180, ATCC119671, ATCC343) using bead-beating and S-trap, before loading them to LC-MS/MS. We managed to identify 30 different proteins, within the same day of analysis, with the highest count being 22 proteins in the ATCC116180 strain. Across all the samples we identified TEM-55, SHV-65, and CTX-M-82, which are all related to ESBL. Based on these results, it can be elucidated that LC-MS/MS possess the potential to replace current AST methods, as resistance proteins were identified within the same day of analysis. However, more extensive research is needed.

7 Acknowlegdement

First and foremost, I would like to thank Allan Stensballe and Thomas Bouet Guldbæk Poulsen for giving me the opportunity to work on this project and helping me throughout. Next, I would also like to show my appreciation for Ditte Bech Laursen for taking the time and assisting me with the laboratory work. I also want to send a thank you to all friends and family, who has supported me on this journey. Lastly, I want to send a special thank you to AquaCombine for funding this project and provide me with the necessary materials.

Sami Shajarat

References

[1] Egorov A.M., Ulyashova M.M., and Rubtsova M. YU. "Bacterial Enzymes and Antibiotic Resistance". In: *ActaNaturae* (2018), pp. 33–48.

[2] A Aitken. "Mass spectrometric techniques". In: *Journal of the American Society for Mass Spectrometry* (1998). DOI: doi:10.1016/S1044-0305(98)00040-3.

[3] Carlos Alvarez, Jaime Labarca, and Mauro Salles. *Prevention strategies for methicillin-resistant staphylococcus aureus (MrSA) in Latin America*. Tech. rep. 2010.

[4] Mounyr Balouiri, Moulay Sadiki, and Saad Koraichi Ibnsouda. *Methods for in vitro evaluating antimicrobial activity: A review*. Apr. 2016. DOI: 10.1016/j.jpha.2015.11.005.

[5] Ramona Barbieri et al. *Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity*. Mar. 2017. DOI: 10.1016/j.micres.2016.12.003.

[6] Blake Bertani and Natividad Ruiz. "Function and Biogenesis of Lipopolysaccharides". In: *EcoSal Plus* 8.1 (Feb. 2018). ISSN: 2324-6200. DOI: 10.1128/ecosalplus.esp-0001-2018.

[7] Christian Blumenscheit et al. "Unbiased antimicrobial resistance detection from clinical bacterial isolates using proteomics". In: (2020). DOI: 10.1101/2020.11.17.386540. URL: https://doi.org/10.1101/2020.11.17.386540.

[8] Wanda C Reygaert. "An overview of the antimicrobial resistance mechanisms of bacteria". In: *AIMS Microbiology* 4.3 (2018), pp. 482–501. ISSN: 2471-1888. DOI: 10.3934/microbiol.2018.3.482. URL: http://www.aimspress.com/article/10.3934/microbiol.2018.3.482.

[9] Chara Calhoun, Harrison R Wermuth, and Gregory A Hall. *Antibiotics*. Tech. rep. 2021. URL: https://www.ncbi.nlm.nih.gov/books/NBK535443/.

[10] *Challenges to Tackling Antimicrobial Resistance*. Tech. rep. 2019.

[11] Yannick Charretier et al. "Rapid bacterial identification, resistance, virulence and type profiling using selected reaction monitoring mass spectrometry". In: *Scientific Reports* 5 (Sept. 2015). ISSN: 20452322. DOI: 10.1038/srep13944.

[12] Tobias Dörr, Patrick J. Moynihan, and Christoph Mayer. *Editorial: Bacterial Cell Wall Structure and Dynamics*. Sept. 2019. DOI: 10.3389/fmicb.2019.02051.

[13] Erika van Eijk et al. "DNA replication proteins as potential targets for antimicrobials in drug-resistant bacterial pathogens". In: *Journal of Antimicrobial Chemotherapy* 72.5 (2017), pp. 1275–1284. ISSN: 14602091. DOI: 10.1093/jac/dkw548.

[14] Ismahen Essaidi et al. "Phytochemical investigation of Tunisian Salicornia herbacea L., antioxidant, antimicrobial and cytochrome P450 (CYPs) inhibitory activities of its methanol extract". In: *Food Control* 32.1 (July 2013), pp. 125–133. ISSN: 09567135. DOI: 10.1016/j.foodcont.2012.11.006.

[15] Dimard E Foudraine et al. *Rapid and Accurate Detection of Aminoglycoside-Modifying Enzymes and 16S rRNA Methyltransferases by Targeted Liquid Chromatography-Tandem Mass Spectrometry*. Tech. rep. 2021. URL: https://journals.asm.org/journal/jcm.

[16] Dimard E. Foudraine et al. "Accurate Detection of the Four Most Prevalent Carbapenemases in E. coli and K. pneumoniae by High-Resolution Mass Spectrometry". In: *Frontiers in Microbiology* 10 (Nov. 2019). ISSN: 1664302X. DOI: 10.3389/fmicb.2019.02760.

[17] Archana Gupta et al. "Extended Spectrum beta Lactamase-producing Klebsiella pneumoniae Infections: a Review of the Literature". In: (2003). DOI: 10.1038/sj.jp.7210973. URL: https://doi.org/10.1038/sj.jp.7210973.

[18] Habboush Y and Guzman N. *Antibiotic Resistance-StatPearls-NCBI Bookshelf Antibiotic Resistance*. Tech. rep. 9. 2021, p. 2021. URL: https://www.ncbi.nlm.nih.gov/books/NBK513277/.

[19] Karim Hayoun et al. "Evaluation of Sample Preparation Methods for Fast Proteotyping of Microorganisms by Tandem Mass Spectrometry". In: *Frontiers in Microbiology* 10 (Sept. 2019). ISSN: 1664302X. DOI: 10.3389/fmicb.2019.01985.

[20] Shrestha J, Zahra F, and Jr P. Cannady. "Antimicrobial Stewardship". In: (2021). URL: https://www.ncbi.nlm.nih.gov/books/NBK572068/.

[21] Buthaina Jubeh, Zeinab Breijyeh, and Rafik Karaman. *Resistance of gram-positive bacteria to current antibacterial agents and overcoming approaches*. June 2020. DOI: 10.3390/molecules25122888.

[22] Matthew Kalp, Cristopher R. Bethel, and Paul R. Carey. "Why the Extended-Spectrum -Lactamases (ESBLs) SHV-2 and SHV-5 are "Hyper-Susceptible" to Mechanism-Based Inhibitors". In: (2009). DOI: 10.1021/bi9012098.

[23] Garima Kapoor, Saurabh Saigal, and Ashok Elongavan. *Action and resistance mechanisms of antibiotics: A guide for clinicians*. July 2017. DOI: 10.4103/joacp.JOACP{_}349{_}15.

[24] Zeeshan A Khan, Mohd F Siddiqui, and Seungkyung Park. "Current and Emerging Methods of Antibiotic Susceptibility Testing". In: (2019). DOI: 10.3390/diagnostics9020049. URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6627445/.

[25] *Landscape of diagnostics against antibacterial resistance, gaps and priorities*. Tech. rep. 2019.

[26] C Lee Ventola. *The Antibiotic Resistance Crisis Part 1: Causes and Threats*. Tech. rep. 4. 2015.

[27] Jingyi Liu et al. "Characterization of phenolic acid antimicrobial and antioxidant structure–property relationships". In: *Pharmaceutics* 12.5 (May 2020). ISSN: 19994923. DOI: 10.3390/pharmaceutics12050419.

[28] Maria Lopes et al. "Halophytes as source of bioactive phenolic compounds and their potential applications". In: *Critical Reviews in Food Science and Nutrition* (2021). DOI: 10.1080/10408398.2021.1959295.

[29] M. Manisha. *Components of Bacterial Cell*. Tech. rep. 2021. URL: https://www.biologydiscussion.com/bacteria/bacterial-cells/components-of-bacterial-cell-7-components-with-diagram/70550.

[30] M. C. MacKinnon et al. *Evaluation of the health and healthcare system burden due to antimicrobial-resistant Escherichia coli infections in humans: a systematic review and meta-analysis*. Dec. 2020. DOI: 10.1186/s13756-020-00863-x.

[31] Maria Miklasińska-Majdanik et al. *Phenolic compounds diminish antibiotic resistance of staphylococcus aureus clinical strains*. Oct. 2018. DOI: 10.3390/ijerph15102321.

[32] Salton MRJ and Kim KS. *Chapter 2 Structure General Concepts Gross Morphology*. Tech. rep. 2021. URL: https://www.ncbi.nlm.nih.gov/books/NBK8477/?report=reader.

[33] Hiroshi Nikaido. *Multidrug resistance in bacteria*. 2009. DOI: 10.1146/annurev.biochem.78.082907.145923.

[34] Jim O'Neill. *Tackling drug-resistant infections globally: final report and recommendations*. Tech. rep. 2016.

[35] Clement Opoku-Temeng, Scott D. Kobayashi, and Frank R. DeLeo. *Klebsiella pneumoniae capsule polysaccharide as a target for therapeutics and vaccines*. Jan. 2019. DOI: 10.1016/j.csbj.2019.09.011.

[36] Bradley R. Parry et al. "The bacterial cytoplasm has glass-like properties and is fluidized by metabolic activity". In: *Cell* 156.1-2 (2014), pp. 183–194. ISSN: 10974172. DOI: 10.1016/j.cell.2013.11.028.

[37] David L. Paterson and Robert A. Bonomo. "Extended-Spectrum β-Lactamases: a Clinical Update". In: (2005). DOI: 10.1128/CMR.18.4.657-686.2005. URL: https://journals.asm.org/doi/abs/10.1128/CMR.18.4.657-686.2005.

[38] Pascale Peeters et al. "The impact of initial antibiotic treatment failure: real-world insights in patients with complicated, health care-associated intra-abdominal infection". In: (2018). DOI: https://doi.org/10.2147/IDR.S184116. URL: https://www.dovepress.com/the-impact-of-initial-antibiotic-treatment-failure-real-world-insights-peer-reviewed-fulltext-article-IDR.

[39] James Pitt. "Principles and Applications of Liquid Chromatography-Mass Spectrometry in Clinical Biochemistry". In: (2008).

[40] Javad Sharifi Rad, Seyedeh Mahsan Hoseini Alfatemi, and Majid Sharifi Rad. "In vitro assessment of antibacterial activity of Salicornia herbacea L. seed extracts against multidrug resistant grampositive and gram-negative bacteria". In: *International Journal of Biosciences (IJB)* (Mar. 2014), pp. 217–222. ISSN: 22206655. DOI: 10.12692/ijb/4.6.217-222.

[41] Simone Rentschler, Lars Kaiser, and Hans-Peter Deigner. "Emerging Options for the Diagnosis of Bacterial Infections and the Characterization of Antimicrobial Resistance". In: *International Journal of Molecular Sciences* 22.1 (2021). ISSN: 1422-0067. DOI: 10.3390/ijms22010456. URL: https://www.mdpi.com/1422-0067/22/1/456.

[42] Andrew Robinson and Antoine M. Van Oijen. *Bacterial replication, transcription and translation: Mechanistic insights from single-molecule biochemical studies*. May 2013. DOI: 10.1038/nrmicro2994.

[43] V Serafim et al. "Detection of Extended-Spectrum β-Lactamase and Carbapenemase Activity in Gram-Negative Bacilli Using Liquid Chromatography – Tandem Mass Spectrometry". In: (2020). DOI: https://doi.org/10.2147/IDR.S267160. URL: https://www.dovepress.com/detection-of-extended-spectrum-beta-lactamase-and-carbapenemase-activi-peer-reviewed-fulltext-article-IDR.

[44] Sibhghatulla Shaikh et al. "Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment". In: *Saudi Journal of Biological Sciences* 22.1 (2015). Special issue: Biological Aspects of Global Health Issues, pp. 90–101. ISSN: 1319-562X. DOI: https://doi.org/10.1016/j.sjbs.2014.08.002. URL: https://www.sciencedirect.com/science/article/pii/S1319562X14000941.

[45] Masaki Shintani, Zoe K. Sanchez, and Kazuhide Kimbara. *Genomics of microbial plasmids: Classification and identification based on replication and transfer systems and host taxonomy*. 2015. DOI: 10.3389/fmicb.2015.00242.

[46] Thomas J. Silhavy, Daniel Kahne, and Suzanne Walker. *The bacterial cell envelope*. 2010. DOI: 10.1101/cshperspect.a000414.

[47] Gloria Del Solar et al. *Replication and Control of Circular Bacterial Plasmids*. Tech. rep. 2. 1998, pp. 434–464.

[48] Raymond T Suhandynata et al. "Mass Spectrometry–Based Detection of Beta Lactam Hydrolysis Enables Rapid Detection of Beta Lactamase Mediated Antibiotic Resistance". In: *Laboratory Medicine* (Aug. 2021). ISSN: 0007-5027. DOI: 10.1093/labmed/lmab068.

[49] Feifei Sun et al. "An integrated data-dependent and data-independent acquisition method for hazardous compounds screening in foods using a single UHPLC-Q-Orbitrap run". In: (2020). DOI: https://doi.org/10.1016/j.jhazmat.2020.123266.

[50] Sonia Szymańska et al. "Bacterial microbiome of root-associated endophytes of Salicornia europaea in correspondence to different levels of salinity". In: *Environmental Science and Pollution Research* 25.25 (Sept. 2018), pp. 25420–25431. ISSN: 16147499. DOI: 10.1007/s11356-018-2530-0.

[51] Tosin Thompson. "The staggering death toll of drug-resistant bacteria". In: (2022). URL: https://www.nature.com/articles/d41586-022-00228-x.

[52] Martin Welker and Alex van Belkum. *One System for All: Is Mass Spectrometry a Future Alternative for Conventional Antibiotic Susceptibility Testing?* Nov. 2019. DOI: 10.3389/fmicb.2019.02711.

[53] OA A. Wintola and AJ J. Afolayan. "The antibacterial, phytochemicals and antioxidants evaluation of the root extracts of Hydnora africanaThunb. used as antidysenteric in Eastern Cape Province, South Africa". In: *BMC Complementary and Alternative Medicine* 15.1 (Sept. 2015). ISSN: 14726882. DOI: 10.1186/s12906-015-0835-9.

[54] Roberta J Worthington and Christian Melander. "Overcoming resistance to β-lactam antibiotics". In: (2013). DOI: 10.1021/jo400236f. URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3644377/.

[55] Alexander Zhivich. "Fighting bacterial resistance: approaches, challenges, and opportunities in the search for new antibiotics. Part 1. Antibiotics used in clinical practice: mechanisms of action and the development of bacterial resistance". In: (2017). DOI: 10.18527/2500-2236-2017-4-1-31-51.