Induced antibiotic resistance and staphyloxanthin as a prospective target for treatment against pathogenic antibiotic-resistant staphylococci

By

Martin Mbugua Mushomba

10066846

Supervisor: Prof JJM Meyer

Submitted in fulfilment of the requirements for the degree

MSc Medicinal Plant Sciences

in the Faculty of Natural and Agricultural Sciences

University of Pretoria

February 2023

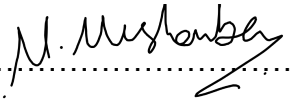
Acknowledgements

I would like to express my gratitude to my supervisor, Prof. Marion Meyer for all his patience and insights as he guided me throughout this project. A profound thanks to Dr. Emmanuel Tshikalange for his assistance and support which made this study possible. I'm also deeply grateful to my friend and colleague Sewes Alberts, a brilliant laboratory 'all-rounder', always keen to help me out of a fix and kindly assisted me in my GC-MS analysis. A special thanks to Dr. Mamoalosi Selepe at the Department of Chemistry who generously shared her expertise in our chromatographic analyses. I am also grateful to all my friends and colleagues on the floor who were always so kind and helpful. Finally, I would like to thank my family who have always supported me. My father Charles Billy Mushomba for his endless words of encouragement and my loving mother Jane Wangui Mushomba for all her prayers.

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Declaration

This thesis is the result of the author's original work except where acknowledged or specifically stated in the text. It has not been submitted for any other degree or examination at any other university or academic institution.



Signed: Martin Mbugua Mushomba (10066846)

Contents

Abstract

The past century saw the discovery of antibiotics as an effective treatment for bacterial infections. Life-threatening infections that were previously untreatable could now be neutralized by antibiotics with few side effects. However, in recent years this once effective treatment has become less efficient as the bacteria it once treated are becoming increasingly resistant. Antibiotic resistance presents a major concern in human public health not just on a national level, but potentially at a global level. The development of bacterial resistance to antibiotics is a natural response to stress.

Plants have always been an important source of medicines and treatments. In cases where bacteria have become resistant to antibiotics, there have been notable successes in re-establishing antibiotic effectiveness when the antibiotics were used in combination with antibacterial plant extracts. The aim of this study was to compare the metabolites of an initially susceptible wild-type (WT) Staphylococcus with its mutated strain which had lost its antibiotic susceptibility. The mutated antibiotic resistant strain was made by exposing the susceptible WT strain to starvation stress. Metabolites from both strains were then extracted in methanol and analysed by ^{1}H-NMR. Multivariate analyses of the NMR spectra showed significant differences in metabolites between the WT and resistant strains. LC-MS and GC-MS analyses of methanolic bacterial extracts showed that staphyloxanthin, a carotenoid that aids in pathogenesis and protects against oxidative stress was partially identified in low concentration from the WT strain. This was confirmed by comparing mass spectra with data on the NIST (National Institute of Standards and Technology) library database. The susceptible WT strain produced two staphyloxanthin precursors, compounds very similar to the two long chain parts of staphyloxanthin, while the resistant strain did not produce any detectable staphyloxanthin or its precursors. The precursors detected by GC-MS analysis and determined from the NIST Library were similar to squalene and tetradecanoic acid. These were the long hydrocarbon chains on the staphyloxanthin structure. This comparative model of analysing a WT and its consequently resistant strain could also be used as a platform to test the effectiveness of different plant treatments against bacterial defence mechanisms like staphyloxanthin. Focusing on the mechanisms of resistance without impeding bacterial growth might reduce the rate of adaptive resistance in-turn, thereby reducing the rate of antibiotic resistance during antibiotic therapy.

Chapter 1: Introductory Chapter

1.1 Introduction

Since early 2020, the world has been under the grip of the SARS-CoV-2 novel coronavirus. However, there is another medical threat that existed before this pandemic that also poses a very serious threat to global health. That threat is the resistance to antibiotics by pathogenic bacteria, a clinical problem that has been identified as a considerable challenge to treating bacterial infections and as such a serious problem in public health (Church and McKillip, 2021; Michael et al., 2014). The beginning of the 20th century, a time in which infectious diseases were the leading cause of death globally, the discovery of various antibiotics was heralded as a ground-breaking event which could revolutionize medicine (Yoneyama and Katsumata, 2006). However, over the years bacteria were increasingly developing resistance to antibiotics. According to the Centers for Disease Control and Prevention (CDC) 2019 report, over 2.8 million cases of antibiotic resistant infections were recorded annually in the United States with over 35,000 fatalities annually (CDC, 2019). In their detailed review of the antibiotic crisis, Ventola (2015) discussed the immense strain that antibiotic resistance infections are causing to health infrastructure and to the economy due to their higher costs of treatment. According to Thorpe et al. (2018), antibiotic resistance increased the cost of treating a bacterial infection by US$1,383 which amounted to an annual national cost of US$2.2 billion based on the 2014 estimate for number of infections. The World Bank projected in their worst-case scenario that the long-term cost of antimicrobial resistance could result in a 3.8% loss in GDP annually by 2050 with an annual shortfall of US$3.4 trillion by 2030 (World Bank Group, 2017). An article in The Economist magazine titled ‘The grim prospect’ stated in relation to the emergence of antibiotic resistant bacterial pathogens that “…the evolution of pathogens is making many problems worse” (The Economist, 2016).

1.1.1 The origins of antibiotics

Antibiotics are generally defined as antibacterial agents, often of biological origin that are largely used to treat bacterial infections (Sandoval-Motta and Aldana, 2016). The first antibiotics of biological origin were isolated by the Scottish scientist, Sir Alexander Fleming in 1929 from the fungus Penicillium rubens. After leaving a Petri dish opened, he observed

bacterial colonies failed to colonise the region around the fungi. He isolated the bactericidal compound which was impeding bacterial growth and named it penicillin after the fungus that was producing it (Fleming, 1929). Fleming (1929) described his newly discovered antibacterial penicillin as an antiseptic. The term 'antibiotic' which means "against life" was first used by Waksman and Woodruff (1942) when distinguishing semi-synthesized antibacterial compounds (like sulphonamides) from antibacterial compounds isolated from microbes like fungi (Yoneyama and Katsumata, 2006). This first use was confirmed by Waksman (1971). Compared to semi-synthetic antibacterial treatments like sulfonamide antiseptics, penicillin was a much safer treatment against bacterial infection (Hassanein, 2019).

Though antibiotics from biological sources are used for killing bacteria, there is a lot of evidence to suggest that these compounds also play a role in the communication between bacteria/fungi and other biological functions (Davies et al., 2006). Antibiotics therefore don't simply kill bacteria but also serve to regulate biological processes in some bacteria.

In the early 1930s, antibiotics were seen as a marvel of modern medicine. In those early years, many previously serious, often fatal infections were easily and effectively treated by administering antibiotics. Bacterial resistance to antibiotics did not emerge as a new concept (Levy and Marshall, 2004), since even as far back as the earliest days of Fleming's work, he had noted that while some bacteria were inhibited by penicillin, there were others that were not susceptible (Fleming, 1929).

1.1.2 The emergence of antibiotic resistance

The first incidences of antibiotic resistance were noted in hospitals as far back as the 1930s when sulphonamide-resistant *Streptococcus pyogenes* was observed in American military hospitals (Selden et al., 1971). In the 1940s, penicillin-resistant *Staphylococcus aureus* was identified in a number of London's public hospitals (Levy and Marshall, 2004). Waksman et al. (1942) who had coined the term 'antibiotic' noted that a culture of microbes grown in the presence of antibiotics can become resistant to higher concentrations of the substance.

A new trend was soon observed in which following the discovery of a new antibiotic, a resistant strain of bacteria was later identified. An example of this was in the discovery of streptomycin in the 1940s, which was almost immediately followed by the emergence of streptomycin-resistant *Mycobacterium tuberculosis* (Levy and Marshall, 2004). Some of the first cases of multi-drug resistance came from *Escherichia coli*, *Shigella* spp. and *Salmonella* spp. bacteria which were identified in the 1960s. These were largely observed in the developing world and for some time, many in the Western World did not take note of it (Levy and Marshall, 2004). Multi-drug resistance, which is the resistance of one bacterial strain to multiple antibiotics, consequently emerged as an important medical concern after a strain of *Klebsiella* showing resistance against several different antibiotics, was isolated in the USA in the summer of 1967 (Selden et al., 1971). This effectively dispelled the rumour that the war against bacterial pestilence had been won (Rogers et al., 2012). If anything, one may be inclined to say that the battle has only just begun.

The first solution to certain antibiotics losing their effectiveness against bacteria would be to find new antibiotics that bacteria would not be resistant to (Ventola, 2015). This is what happened during the course of the 20th century as new antibiotic classes were being discovered and new antibiotics were being isolated from biological sources (Kirby, 1950). However, this approach did not solve the problem completely as bacteria would soon develop resistance to these antibiotics (CDC, 2019; Ventola, 2015). The same trend kept on repeating with a new antibiotic being introduced with initial success against pathogenic bacteria and then some bacteria slowly lose sensitivity to the antibiotic until there is a large group of bacteria that are resistant to a list of antibiotics (Uddin et al., 2021). Antibiotic resistance is a natural process as bacteria will naturally evolve better ways to survive (Rodriguez-Rojas et al., 2013). The initial effectiveness and consequent mass production of antibiotics resulted in their wide and indiscriminate use in the clinical setting as well as in agriculture. This has significantly increased the rate at which antibiotic resistance develops and spreads (Ventola, 2015). The higher incidence of antibiotic resistant pathogenic bacteria showed that we might be on the verge of a “post-antibiotic era” in which virtually all pathogenic bacteria will have become permanently resistant to the antibiotics that were previously used to treat them (Laxminarayan et al., 2013).

1.1.3 COVID-19 and antibiotic resistance

The SARS-CoV-2 virus has put significant pressure on a health system that was already struggling to contend with drug resistant infections like *M. tuberculosis* (Chisompola et al., 2020). While patients who were admitted to hospitals with COVID-19 were more vulnerable to infection and succumbed to bacterial pneumonia, it has been predicted that COVID-19 will not increase the spread of antibiotic resistance. In wealthier countries with more effective health systems and facilities, the increased use of disinfectants and handwashing is predicted to decrease the spread of opportunistic infectious bacteria like *Enterococci* spp. and *S. aureus* (Collignon and Beggs, 2020).

Viral respiratory infections like that of COVID-19 often comes with bacterial co-infections which can be a significant cause of morbidity and mortality. The few studies that have been carried out and published have shown a low rate of bacterial co-infections in patients infected with the COVID-19 virus (Alder et al., 2020; Mahmoudi, 2020). However, information on COVID-19 patients is currently limited. There is still a threat of COVID-19 patients being exposed to hospital-acquired antibiotic resistant bacteria when undergoing treatment in hospitals. The COVID-19 pandemic is not expected to worsen the antibiotic resistance crisis due to several factors such as the decline in hospital patients due to more patients being encouraged to stay home and limitations in travel could reduce the spread of antibiotic resistance strains. However, the COVID-19 measures restricting the movement of people are expected to temporarily contain the spread of antibiotic resistance which is projected to return to its pre-COVID levels as more COVID measures are rolled back (Livermore, 2021).

1.2 Objectives and hypothesis

Antibiotic resistant and antibiotic susceptible bacteria often share a lot of similarities, which makes the treatment of antibiotic resistance more complicated. Finding differences between resistant and susceptible strains could help reduce treatment periods and minimize the spread of antibiotic resistance (Mitsakakis et al., 2018). The aim of this study was to compare the metabolites of an initially susceptible wild-type (WT) *Staphylococcus xylosus* with its mutated strain which had lost its antibiotic susceptibility. This species was selected for the study

because it is non-pathogenic, often used as a starter-culture and easy to grow (Rosenstein and Götz, 2013).

It is hypothesised that this investigation may identify a unique metabolite in antibiotic resistant bacteria that is not present in susceptible strains which could help in the treatment or diagnosis of antibiotic resistance.

The objectives of this study were;

- To create an antibiotic resistant strain from an antibiotic susceptible WT.
- Compare the metabolomic profiles of the susceptible and consequently resistant strains.
- Isolate and identify potential biomarkers or new targets of an antibiotic resistant bacterial strain.

It was postulated that the antibiotic susceptible strain would become resistant to antibiotics after exposure to stress. The consequently resistant mutant would then produce new metabolites in order to acquire antibiotic resistance. It was also hypothesised that certain biomarkers might be identified during this comparative study which might be useful in the diagnosis of antibiotic resistant bacteria.

Chapter 2: Antibiotics and Antibiotic Resistance

2.1 Introduction

2.1.1 The origin of antibiotics

Contrary to popular belief, the history of antibiotic use for medicinal purposes goes back much further than the discovery of penicillin in the 20th Century. There has been strong historical evidence that some 'modern' antibiotics may have been used by certain ancient civilizations. Among these are traces of the antibiotic tetracycline that has been identified by mass spectrometric analyses of human bone fossils from ancient Sudan dating back to around 350-550 CE (Nelson et al., 2010).

Tetracycline was also present in skeletal fossilized remains found in Egypt dating from the late Roman period. The use of tetracycline in these sources may have coincided with the lack of records showing infectious diseases amongst the Sudanese Nubians at the time (Cook et al., 1989). The reason tetracycline could be detected by mass spectrometry over other antibiotics such as penicillin was owed to its chelation that allowed for its incorporation into bone and enamel (Aminov, 2010). There may be other antibiotics used in ancient remedies whose remains may have been otherwise undetectable. Aminov and Mackie (2007) have discussed the possibility that these ancient uses of antibiotics may have had an influence in the development of antibiotic resistance well before its modern discovery and use.

The conceptualization of modern antibiotic therapy can be associated with the prominent German physician Paul Elhrich (1854-1915) who conceived the idea of specialised chemotherapy targeting only the infectious agent causing disease. He considered this specific chemical treatment as a "magic bullet", one that would destroy all the pathogens causing infection without harming the host. While doing research on trypanosomal infections, Elhrich embarked on a bold project in 1903 to systematically screen hundreds of different chemical compounds against murine trypanosomal infections. This led to the discovery of arsphenamine in 1909 (the 606th compound Ehrlich tested), which was the first effective treatment against the pathogenic spirochete *Treponema pallidum* which causes syphilis (Riethmiller, 2005; Bosch and Rosich, 2008).

Ehrlich's approach established the basis of drug discovery strategies in the pharmaceutical industry. This led to the discovery of a list of sulphonamide-based antibacterial compounds that came before the discovery of penicillin. Sulphonamides are generally considered to be the oldest class of synthetic antibiotics (Aminov, 2010).

The discovery of penicillin, the first isolated antibiotic, was heralded as possibly the greatest medical achievement of the 20th century. The 1945 Nobel Prize in Medicine was shared by Alexander Fleming who first isolated the compound and chemists Howard Florey and Ernst Boris Chain who developed the synthesis of penicillin following Fleming's isolation of the compound (Lee Ligon, 2004).

In his publication on May 10th 1929, Alexander Fleming famously described how he discovered a mould that contaminated culture-plates that he left open were able to restrict the growth of bacteria cultures. He concluded that the mould had been producing a bacteriolytic substance that would diffuse into the medium around the mould and prevented the growth and proliferation of the different bacteria (including common pathogenic bacteria). He referred to the mould's antibacterial filtrate as *penicillin*, named after the *Penicillium* fungus from which it was isolated (Fleming, 1929).

In clinical practice, Fleming (1929) observed that penicillin was a much more effective antibacterial when compared to other chemical antiseptics at the time, particularly due its complete inhibition of a number of important pathogenic bacteria. These included staphylococci, streptococci, gonococci, meningococci and *Corynebacterium diphtheriae* which were all treatable with penicillin (Chain, 2005). What was even more ideal was that unlike the toxic chemical antibiotics of the time (like sulphonamides and arsphenamine derivatives) penicillin was essentially non-toxic and non-irritant when administered in its minimum effective concentration (Fleming, 1929). This could very well have been the "magic bullet" that Ehlrich had envisioned. The discovery of penicillin brought a new sense of optimism that had previously seemed impossible. The idea that the infections and sicknesses that had plagued humanity for millennia could now be a thing of the past.

However, as noted in Chapter 1, the optimism of these early years following the discovery of penicillin proved to be short lived. Indeed, many of the infections that were initially

treatable by the new miracle drug were observed to quickly develop resistance against penicillin and other antibiotic treatments.

2.1.2 The crisis of clinical antibiotic resistance

With the discovery of effective chemotherapeutics against infectious bacterial diseases, the prominent advances in medicine and science allowed the widespread treatment against a list of initially untreatable infections. Though initially very successful, the pathogenic bacteria that were initially responsive to antibiotic treatment began to develop resistance.

The emergence of clinical antibiotic resistance to penicillin was actually recorded in the same city that penicillin was first isolated only a few years before the Nobel Prize in Medicine was awarded for its discovery. It was noted that a strain of penicillin-resistant *S. aureus* was identified in London civilian hospitals as early as the 1940s (Barber, 1948). Furthermore, antibiotic resistance wasn't only restricted to penicillin. Streptomycin, a bacterial produced antibiotic that was developed after penicillin was also found to be ineffective against some strains of community-based *Mycobacterium tuberculosis* (Crofton and Mitchison, 1948).

Antibiotic resistant *Escherichia coli, Shigella* spp. and *Salmonella* spp. emerged in the late 1950s and early 1960s and caused crises particularly in the developing world (Levy and Marshall, 2004). It was only in the 1970s that antibiotic resistance was officially recognized as a serious health concern by many in the developed countries after infectious bacteria resistant to ampicillin, chloramphenicol and tetracycline (the newest antibiotics of the time) were discovered (Levy and Marshall, 2004).

The development of resistance has only continued to grow over the years. Multidrug resistant (MDR) infections emerged in the 1980s with strains of *Mycobacterium tuberculosis* simultaneously showing resistance against multiple antibiotics. This complication, coupled with the HIV/AIDS pandemic only served to worsen an already dreadful clinical crisis. The most common opportunistic infection in HIV positive patients and the greatest cause of death among these patients in South Africa is tuberculosis (TB).

In a country with the largest number of HIV infected persons in the world, this makes MDR TB a particularly serious threat to public health (Gandhi et al., 2006). Despite the apparent success of antiretroviral (ARV) therapy reducing the incidence of symptomatic TB, HIV-infected patients receiving ARV treatment are still more than five-times at risk of developing tuberculosis compared with non-HIV infected persons and are far more vulnerable to MDR TB. When comparing first-line treatment courses for drug resistant TB, second-line treatment for MDR tuberculosis (requires a longer course) is more toxic, more costly, and is not readily available in resource-limited, under-funded hospitals (Gandhi et al., 2006).

Tuberculosis was the most common cause of morbidity and mortality in HIV infected patients in sub-Saharan Africa at the beginning of the 21st century (Mukadi et al., 2001). Furthermore, it is estimated that around 80% of patients admitted with symptomatic TB in the KwaZulu-Natal are also HIV positive. The effects of MDR *M. tuberculosis* have been reflected in the mortality rates of HIV positive TB patients which is estimated to be as high as 40% per year (Gandhi et al., 2006). Confronting the scourge of HIV/AIDS will require an equal effort in confronting antibiotic resistant TB if the pandemic is to be effectively treated (De Cock and Marston, 2005).

Antibiotic resistance has been more prevalent in the developing world than in the developed world (North America and Western Europe). While the generally more advanced medical systems in the developed world are often able to respond more effectively against pathogenic infections, antibiotic resistance remains a serious threat to public health in North America and Western Europe (Allegranzi et al., 2011).

After the introduction of penicillin in the 1940s, it was widely used in hospitals to treat opportunistic infections like those caused by *S. aureus*. While the antibiotic proved to be highly effective at first, strains of a penicillin-resistant *S. aureus* began to emerge in the 1950s (Fairbrother, 1956). Penicillinase, a β-lactamase enzyme that breaks down penicillin by hydrolysing its β-lactam ring, was identified in *S. aureus* mutants (Novick, 1963). In response, scientists developed a β-lactam antibiotic that could not be hydrolysed and broken by penicillinase. This new antibiotic was named methicillin and was particularly

effective in treating penicillin-resistant *S. aureus*. Shortly after the development of methicillin, strains of methicillin-resistant *S. aureus* (MRSA) were isolated showing the immense difficulty in controlling antibiotic resistance. Numerous strains of MRSA have since been isolated and have been associated with resistance to many other antibiotics (Duckworth, 1993). It was estimated by Rubin et al. (1999) that the cost of treating methicillin resistant *S. aureus* (MRSA) was three times the cost of treating methicillin-susceptible *S. aureus* infections and that the difference went into the several millions of dollars in extended antibiotic courses and other secondary treatments.

According to the American Centre for Disease Control and Prevention (CDC) at least 2 million people in the United States are infected with antibiotic resistant microorganisms each year of which about 23,000 die as a result of the infection (CDC, 2013). In the USA and the UK, it is estimated that 40-60% of all nosocomial *S. aureus* infections are multi-drug resistant. Attempts to treat other nosocomial antibiotic-resistant infections can take many combinations of different antibiotics before an effective antibiotic treatment is identified (Levy and Marshal, 2004).

Nosocomial antibiotic resistance, the occurrence and proliferation of antibiotic resistant infectious bacteria within a hospital setting is particularly concerning. Hospitals are key areas at which infectious bacteria and antibiotics interact with high frequency. As noted earlier, some of the first incidences of antibiotic resistant pathogenic bacteria were observed in hospitals. The factors enhancing the spread of antibiotic resistance, such as over-usage of antibiotics are quite common in hospitals (Goldmann and Huskins, 1997).

Hospitals have been noted as being notorious for the misuse and overuse of antibiotics, which increases the selection of antibiotic resistant strains. Indeed, it was noted by Goldman and Huskins (1997) that many hospital physicians misuse antibiotics by misdiagnosing or over-diagnosing bacterial infections. In particular, Goldmann and Huskins (1997) cited in their review that health practitioners' continuously resorting to broad-spectrum antibiotics has facilitated resistance, particularly amongst Gram-negative bacteria. The convergence of many bacterial pathogens with a variety of antibiotic resistant traits in hospitals means that there is

a large number of resistance genetic variants (Levy, 1998). This makes treatment of emerging nosocomial antibiotic resistance quite challenging.

Figure 2.1 is a schematic representation of the tracking of antibiotic resistance, and its re-emergence over the years. It also shows an incident reported by Meyer et al. (1993) of the resistance to imipenem.

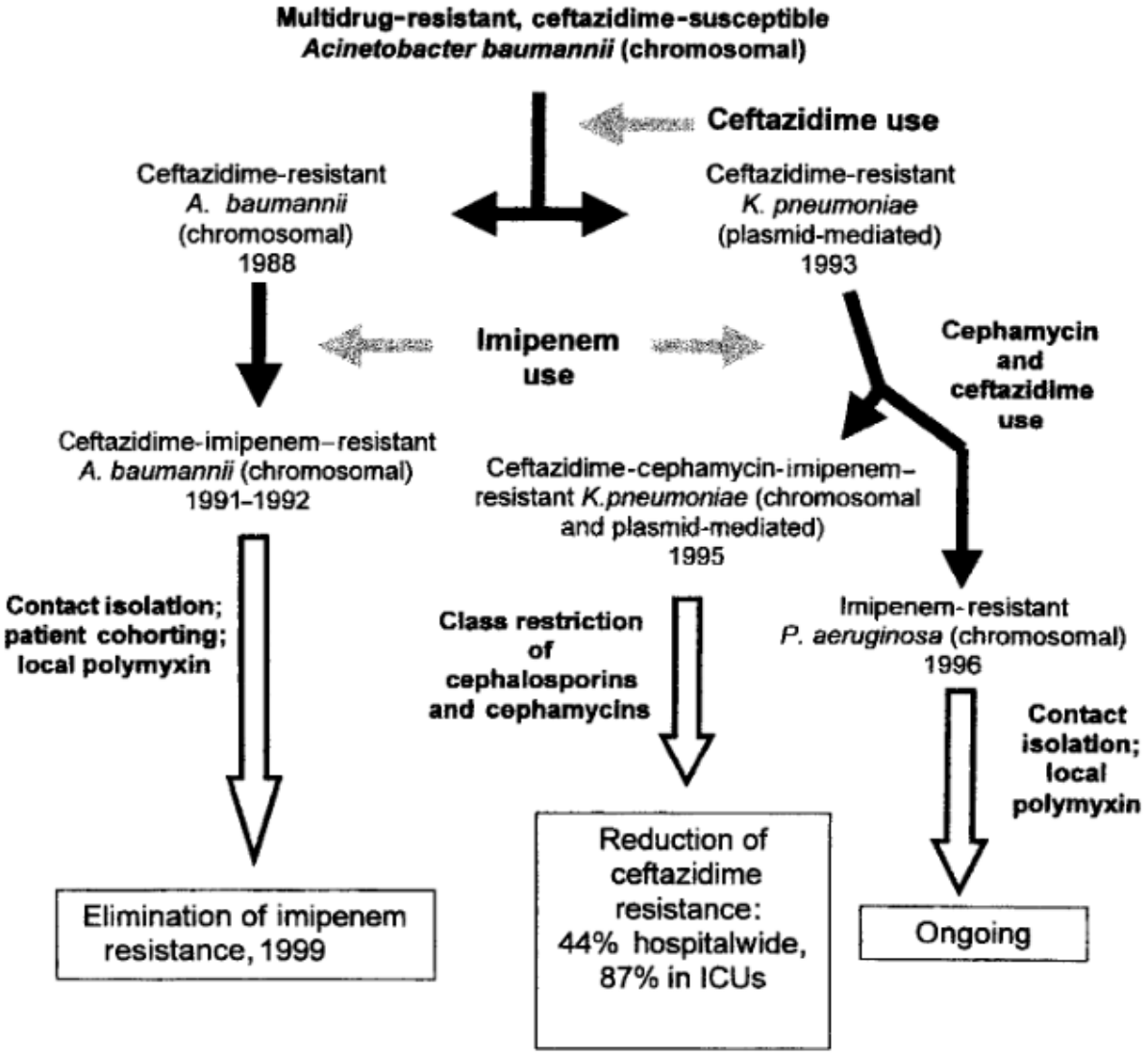


Figure 2.1: Evolution and control of antibiotic resistance among Gram-negative bacilli at New York Hospital Queens. Grey arrows show the initial use of the antibiotic, the black arrows show the first emergence of antibiotic resistance to the drugs, and the white arrows show the consequent re-emergence of antibiotic resistance which is still an ongoing process (ICU, intensive care unit) (Urban et al., 2003).

While there has been some progress in the elimination and control of nosocomial antibiotic resistance (Figure 2.1) new resistant strains continue to emerge. Many hospitals today have

become reservoirs for the emergence and dissemination of antibiotic resistant bacteria. An example is the spread of *Salmonella typhimurium* that was identified as having been spread by discharged patients from a hospital in Brazil that was cited by Goldmann and Huskins (1997).

The spread of antibiotic resistance in hospitals isn't solely due to negligence of medical practitioners. Certain nosocomial infections are not easily diagnosed in the hospital setting, and misdiagnosis can be attributed to the complex progression of bacterial infections (Goldmann and Huskins, 1997). For example, the bacterial colonization of breathing apparatus cannot be easily determined and has been noted as the cause of nosocomial pneumonia. General hygiene standards aren't always sufficient for controlling nosocomial bacteria. Many standard soaps can't remove persistent bacteria unless they have active disinfectants added. Thus, simply following standard procedures doesn't always eliminate infectious nosocomial bacteria (Goldmann and Huskins, 1997). Also, while many hospitals are struggling to control nosocomial bacterial pathogens, the issue grows more serious in the developing world where many hospitals are understaffed and underfunded which is a significant contributing factor to the spread of antibiotic resistance (Goldmann and Huskins, 1997).

A recent study by Chng et al. (2020) in Singapore attempted to map out the opportunistic pathogenic bacteria's antibiotic resistance in hospitals. This was the first study of its kind attempting to characterize nosocomial bacteria resistance genes (or resistomes) from multiple sites in a tertiary hospital environment. They also studied mobile genes (mobilomes) and discovered a large variety of previously unexplored mobile resistance genes which in turn give rise to a variety of combinations of multidrug resistance across different hospital environments. These hospital strains and resistomes were very different to other urban strains of antibiotic resistance. While Chng et al. (2020)'s study could be very useful in tracking the spread of resistance genes, it also reveals how hospital environments are fostering huge reservoirs of multidrug resistance genes. These authors also determined that the multidrug resistant colonies could persist in hospitals for up to eight years.

As more people are being hospitalized during the COVID-19 pandemic, the increased activity could spread these variants and determinants further. Besides resistance genes, there are also genes for disinfectant resistance which are much less understood (McCarlie et al., 2020). Disinfectant resistance genes are shared along with resistance genes making it harder to

eliminate multidrug resistant bacteria from hospital environments by using disinfectants. In fact, the increased use of disinfectants only allows for more disinfectant resistant bacteria to proliferate. This is because using stronger disinfectants kills off more bacteria leaving resistant bacteria to grow with less selective competition in space and nutrients (Madigan et al., 2009).

The seriousness of nosocomial antibiotic resistance culminates as a perfect storm; public hospitals have a high use of antibiotics and disinfectants, they receive patients infected by a variety of antibiotic resistant bacteria and lastly, they are filled with immune-compromised patients such as newborn, elderly, infirm and terminally ill patients. SARS-CoV-2 has been exacerbating co-morbidities and in turn could put most of these already vulnerable patients at an even higher risk. The increased use of disinfectants with the COVID-19 pandemic could also result in an increase in disinfectant resistance making it harder to eliminate resistant bacteria colonies in hospitals. All this creates a convergence of multiple factors that intensify the seriousness of nosocomial antibiotic resistance as possibly the most threatening issue in modern healthcare and medicine (Krzowska-Firych, 2014).

2.1.3 Why do bacteria develop antibiotic resistance?

The sharp increase in antibiotic resistance over the years is often considered to be an abnormal occurrence by current generations that were born in this antibiotic era. A prevalent theme in many publications is that antibiotic resistance is caused by the overuse/misuse of antibiotics, which is itself a significant cause of wide-scale antibiotic resistance (Cusini et al., 2010). On the grander scale however, such notions might seem to disregard the fact that antibiotic resistance is in fact a common occurrence and trait in bacteria (Rodriguez-Rojas et al., 2013).

While bacteria have various responses to external stresses that threaten their survival (including antibiotics) it was extensively discussed by Aminov (2009) amongst others that antibiotic resistance is very likely a natural bacterial process. This was most notably due to the prevalence of antibiotic resistance genes in bacteria that were far removed from the influences of the human clinical setting. An example is the β-lactamase genes which confer resistance against β-lactam bacteria (e.g., penicillin and methicillin) that were identified by structure-based phylogenetic reconstruction of the bacterial evolutionary process showing

that the antibiotic resistant enzymes were first formed billions of years ago. In fact, the genes for serine β-lactamases have been present on plasmids for millions of years (Hall and Barlow, 2004). Indeed, a diverse collection of β-lactamase genes have been identified in bacteria growing in far off Alaskan soils where there is no exposure to the overused/misused causes of antibiotic resistance (Allen et al., 2009).

Aminov (2009) discussed the history of antibiotic resistance which he defined as having two distinctive periods. The first is the macro-evolutionary period, alternatively called the pre-antibiotic era which was when antibiotic genes were developed as part of the bacterial evolutionary development over billions of years. The second is the micro-evolutionary period, which can be alternatively considered as the 'antibiotic era' or the period of humans using antibiotics to treat bacterial infections. This has been driven by the exertion of selective pressure and the horizontal transfer of mobile antibiotic resistance genes (Aminov, 2009).

The long history of antibiotic resistant genes in bacteria may suggest that these genes are actually responsible for serving other purposes in bacteria, besides defence against antibiotics. β-lactam antibiotics are for example, produced by many bacteria which does give strong reason to postulate that they may serve a purpose in ecological communication amongst different bacterial cells. A possible example could be carbapenems, a class of β-lactam antibiotics that are produced by a large number of different bacteria (Aminov, 2009). It was noted that the biosynthesis of structurally similar carbapenem by streptomycetes bacteria and a variety of Gram-negative bacteria was dependent on the same quorum sensory (QS) signallers and factors. Hence, these bacteria respond to the same external QS factors to produce very similar antibiotics which may be used as second-level signalling molecules. It was shown that the antibiotic imipenem initiated global gene expression such as β-lactamase and alginate production when introduced to *Pseudomonas aeruginosa* (Bagge et al., 2004).

Micro-evolutionary antibiotic resistance may have initiated a prior exposure to antimicrobials for bacteria in the 20th century, considering that antibiotics have been used by humans long before this time (Nelson et al., 2010). Antimicrobial activity may have been available in other herbal medications used in traditional remedies. As noted earlier, the

development of antibiotic resistance can be exasperated by selective pressures caused by the regular use of antimicrobial chemotherapies (Cusini et al., 2010). Thus, it is likely that during the long history of antibacterial folk medicines, bacteria that were exposed to these treatments may have begun to develop and promote resistance against these early human chemotherapeutic applications. This may have contributed to the accumulation of antibiotic resistance activity in many common pathogens before the dawn of the antibiotic era in the 20th Century (Aminov, 2007).

2.1.4 Mechanisms of resistance

As discussed above, bacteria have a long history of evolutionary development and genetic variation. Thus, the molecular mechanisms that they often utilize to overcome antibiotics are immensely complex and intricate biochemical processes reflective of the billions of years of their specialization in surviving.

Owed to the different chemical compositions and activities of the different classes of antibiotics, bacteria will often employ different modes of resistance against the different antibiotics.

Table 2.1 (Singh, 2013) provides a basic overview of the various modes of resistance generally used against different antibiotics by bacteria. The resistance mechanisms of bacteria are diverse owed to the genetic diversity of many bacterial species and their evolutionary development in surviving. The main mechanisms of bacterial resistance to antibiotics are shown in Table 2.1. By means of these cellular processes, bacteria can not only survive, but proliferate in the presence of potent bactericidal antibiotics that had previously been effective in inhibiting their growth. The level at which these antibiotic mechanisms are occurring in bacterial populations is extremely high. The problem of antibiotic resistance is made by the ease at which bacteria are able to spread the determinants conferring these mechanisms to other bacteria.

Table 2.1: Antimicrobial drugs and their source of resistance (Singh, 2013).

Drug class with examples	**Mode of Resistance**	**Effective against**
Polypeptides e.g.: Bacitracin	Inhibition of cell wall synthesis	Gram-positive bacteria
β lactams e.g.: Penicillin Cephamycin	Inhibition of cell wall synthesis, efflux, altered target	Gram-negative bacteria
Cyclic peptides e.g.: Gramicidins	Alter cytoplasmic membranes	Gram-positive bacteria
Polypeptides e.g.: Polymixin		Gram-positive bacteria particularly *Pseudomonas*
Tetracyclins e.g.: Doxycycline Tetracycline	Efflux pump	Broad spectrum effective against gram-positive and gram-negative bacteria and also mycoplasma
Phenicols e.g.: Chloramphenicols Aminoglycosides e.g.: Gentamicin Kanamycin Neomycin	Mutation in active target sites, efflux	Broad spectrum bacteria
Macrolides e.g.: Azithromycin Erythromycin Telithromicin	Target modification due to enzymatic degradation or modification, efflux	Broad spectrum bacteria
Pyrimidines e.g.: Trimethoprim	Target bypass, efflux	Broad spectrum bacteria, some protozoa and fungi

The acquisition of genes is greatly enhanced by the efficient gene transfer systems that bacteria naturally employ, including plasmids, integrin systems and active transposable elements. These can transport antibiotic resistance determinants amongst different bacterial cells. By a large measure, these antibiotic resistance genes are conveyed from antibiotic resistant bacteria to antibiotic susceptible bacteria via bacterial plasmids (Bennett, 2008). Through genetic transfer, a variety of these resistance mechanisms listed

in Table 2.2 can be acquired by previously antibiotic susceptible bacteria (Kumar and Varela, 2013).

Table 2.2: Mechanisms of antibiotic resistance (Kumar and Varela, 2013).

Basis of resistance	**Mechanism of resistance**	**Target**	**References**
Enzyme	Hydrolysis	β-lactamases Esterase C-P lyase complex	(Davies, 2012) (Philipon et al., 1989)
	Group transfer	Acetyltransferase Phosphotransferase Nucleotidyltransferase Glycosyltransferase Ribosyl transferase Thiol transferase	(Wright, 2005)
	Redox process	TetX	(Volkers et al., 2011) (Fosberg et al., 2015)
Target modification	Structural Alterations/ modifications Mutations in Amino acid substitutions Methylation Mutation	Penicillin binding proteins Cell wall precursors genes Ribosomal subunits RNA polymerase DNA gyrase/topoisomerase 16S rRNA 23S rRNA 23S rRNA	(Robicsek et al., 2009) (Fischer et al., 2005).
Reduced permeability	Reduced expression/defective protein	Porins	(Delcour, 2009)
Target protection	Ribosome protection	Ribosome protection	(Roberts, 2005)
Efflux	Active extrusion	Membrane proteins	(Webber and Piddoc, 2003)

2.1.4.1 Enzymatic drug inactivation mechanism

Bacteria produce antibiotic-specific enzymes that render antibiotics inactive by hydrolysis, group transfer and redox reactions (Davies, 2012; Kumar and Varela, 2013). One such method already discussed is that of β-lactamase enzymes that hydrolyse the β-lactam ring of penicillin and other β-lactam antibiotics. It has been shown that of β-lactamase genes have been present in bacteria for millions of years. These genes that express β-lactamases (*bla*) are believed to be carried on the bacterial chromosome (e.g., AmpC β-lactamase) and on plasmids. The first *bla* genes discovered on plasmids were the genes for TEM-1 β-lactamase in a strain of *Escherichia coli* (Datta and Kontomichalou, 1965). The second *bla* genes discovered were the plasmid-borne genes of SHV-1 (sulfhydryl variable active site) β-lactamase (Livermore, 1995). TEM and SHV enzymes have been observed to have the ability to hydrolyse a broad range of extended spectrum cephalosporins and other β-lactam antibiotics. Such enzymes are collectively known as extended spectrum β-lactamases (ESBLs) (Philipon et al., 1989).

Another enzymatic mechanism of antibiotic inactivation is the structural alteration of the drug by adding on a different functional group, like acyls, ribosyl, phosphoryl or thiol groups (Wright, 2005). This reaction is often irreversible thus the neutralised antibiotic molecule loses its specific target binding ability due to the structural change. Antibiotics that are generally neutralised by this mechanism include aminoglycosides, fosfomycin, macrolides, lincomycin and chloramphenicol (Davies, 2012).

There have been a large variety of tetracycline-degrading enzymes that inactivate tetracycline, which have been found to be the most common enzyme that degrades natural antibiotics. A family of flavoenzymes was recently discovered by Fosberg et al. (2015) in soil cultures and observed to be instrumental in conferring bacterial resistance against the antibiotic, particularly in *E. coli* (Volkers et al., 2011; Fosberg et al., 2015).

2.1.4.2 Target modification and protection

Bacteria can alter the antibiotic target such that the antibiotic loses its effective bactericidal properties as it is unable to bind to its target. One such example common with

staphylococcal antibiotic resistance is the alteration of the penicillin binding protein (PBP), the actual cellular target for β-lactam antibiotics. *S. aureus*, which has been discussed above as causing serious nosocomial infection, has the ability to change the shape of its PBPs such that penicillin and methicillin have a reduced binding and thus reduced effectiveness. This is often characterized by an over expression of the variant PBPs (Fischer et al., 2005).

Bacterial enzymes also undergo modification, often by substituting amino acids in what is known as the quinolone-resistance determining region (QRDR) of DNA gyrase and topoisomerase IV targets quinolone antibiotics. This significantly reduces the binding of quinolone antibiotics and is responsible for the high incidence of quinolone resistance among many virulent Enterobacteriaceae (Robicsek et al., 2009).

Another bacterial mechanism of resistant bacteria is to conceal the target from the antibiotic. These include ribosomal protection mechanisms. This is effective against antibiotics such as tetracycline which functions as a bacterial protein synthesis inhibitor. The bacteria produce special ribosome protecting proteins that bind to the ribosomal target and prevent tetracycline from binding and inhibiting the ribosome (Roberts, 2005).

2.1.4.3 Reduced permeability

This mechanism involves the reduction of antibiotic molecules entering or being taken up into the bacterial cell. A common mechanism is the reduced expression of porin channels spanning the cell membrane and outer membrane that allow small molecular weight molecules to enter the cell (Delcour, 2009). These channels are often used by antibiotics to enter the bacterial cells. When bacteria reduce the expression of these outer membrane porins, they don't integrate into the outer membrane or become non-functional thus rendering these channels unusable. This restricts the entry of antibiotic compounds reducing their effectiveness (Pages et al., 2008). Clinically important bacterial pathogens which have incidentally been prioritized as the most threatening to public health by the World Health Organisation (WHO, 2017) have been found to use this mechanism. These include *Serratia marcescens Salmonella enterica, Enterococcus aerogenes, Klebsiella*

pneumoniae, and *P. aeruginosa*. Most antibiotics are hindered by this mechanism, including β-lactams, fluoroquinolones, aminoglycosides, and chloramphenicol (Delcour, 2009).

2.1.4.4 Active drug efflux

The last mechanism to be discussed is the use of active efflux pumps. It is most likely the most common of the antibiotic resistance mechanisms owed to its versatility and effectiveness in reducing antibiotic concentrations in the cells by removing large molecules from the inside of bacterial cytoplasm. Many antibiotic resistant bacteria express a large amount of these efflux pumps. The two main types of active efflux pumps are the first type which have a primary active transport i.e., utilizes the hydrolysis of ATP to actively efflux (pump out) drugs from cells. The second type is said to have a secondary active transport, using an ion gradient for active drug efflux from cells (Webber and Piddoc, 2003).

The most common ATP driven transporters are also known as ABC (for ATP-binding cassette) or P-glycoprotein transporters and are dependent on ATP as an energy source to help the transporters pump compounds in the cytoplasm against the concentration gradient. The second type of efflux pumps operate on different variants and are effective against a wider range of antibiotics. These systems include tetracycline efflux pumps and multidrug efflux pumps of the major facilitator superfamily (Kumar and Varela, 2013).

In conclusion, this particular resistant mechanism hasn't been proven to be the most ideal in understanding drug resistance as recent research in resistance mechanisms suggest that not all interactions of bacteria with antibiotics can be reduced to these frames reflecting a classical bullet-target concept. An example is the more recent work on novel antibiotic resistance mechanisms which postulated that a "kin selection" concept might be more prevalent (Aminov, 2010). This means that not all bacteria in the population develop mechanisms of resistance, rather a set number in the population develop communal systems of resistance which are in turn used to protect the larger population (Lee et al., 2010). It could well be argued that this system often observed in the macro-organismal world may seem to be just as applicable to the microbial world. Such communal systems

have been noted in biofilms which have shown to be immensely effective against antibiotics and other population threats to bacterial communities (Rogers et al., 2012).

2.1.4.5 Persistence mechanisms

In addition to the mechanisms of antibiotic resistance, bacteria also have additional ways of increasing their antibiotic tolerance. This is known as persistence and it is achieved by metabolic versatility, which is an ability to quickly change their metabolism while under stresses that impede their growth. Persistence is an epigenetic trait which means that unlike the mechanistic antibiotic resistance which results in genomic changes, persistence does not reflect a change in the bacterial DNA sequences (Debbia et al., 2001; Kussel et al., 2005).

Persistence was first observed by Bigger (1944) who noted that certain bacteria could tolerate an antibiotic concentration which had killed other bacteria. He noted that a few of the bacterial cells would “persist” even after antibiotics (Bigger, 1944). Persistence can result in the formation of “persister cells” or even the formation of spores (Eichenberger et al., 2004) that are able to become dormant when under stress and grow when conditions are favourable.

Persistent cells grow slower and are able to survive different stresses in different ways. Discussing persistence in staphylococcal bacteria, Onyango and Alreshidi (2018) explained different adaptative responses that help staphylococci persist under environmental and clinical settings. They often come as a modification to the cell as a response to external stress. Examples include temperature-induced modifications, osmotic pressure, induced modifications, and nutritional adaptation when in nutrient-limited environments etc. (Onyango and Alreshidi, 2018). Antibiotics can also induce antibiotic tolerance after exposure to sub-lethal concentrations of antibiotics, which can do more damage in promoting resistance than at lethal concentrations (Andersson and Hugh, 2012).

2.2 Plant antimicrobials

2.2.1 The need for alternative treatments against antibiotic resistance

The progressive development of antibiotic resistance during the 20th and 21st century has been driven by the transfer of a variety of genetic determinants that have caused a great crisis in public health. Currently, many bacterial pathogens associated with important clinical infections have developed multidrug-resistant (MDR) strains in which synthetic antibiotic chemotherapeutics and their subsequent overuse have been a driving factor. A very real threat is MDR *Mycobacterium tuberculosis* which is a major problem in South Africa as mentioned above, particularly amongst HIV infected patients as mentioned previously (Gandhi et al., 2006).

Numerous other infectious strains have shown to be MDR, several which are nosocomial pathogens that have been prioritised by the WHO (2017) such as *Acinetobacter baumannii, Campylobacter jejuni, Enterobacter* spp., *Enterococcus faecium, E. faecalis, Escherichia coli, Haemophilus influenza, K. pneumonia, Proteus mirabilis, P. aeruginosa, Streptococcus pneumonia*, *Salmonella* spp. and *Serratia* spp. A number of pathogenic staphylococcus strains are also becoming MDR including *Staphylococcus aureus* and *S. epidermidis* (Davies and Davies, 2010).

These MDR pathogenic bacteria often cause a higher mortality and more abrasive morbidity in infection. They are often more pathogenic due to acquiring a large amount of specialized pathogenic genetic determinants making them dreadfully virulent and fatal in infection. Antibiotic resistance mechanisms aid in pathogenic invasion and are thus virulence factors. The more mechanisms of resistance a certain pathogen can effectively deploy, the more virulent it becomes (Davies and Davies, 2010). These kinds of pathogens are colloquially referred to as “superbugs” and are often resistant against most antibiotics. Thus, the infections associated with these “super bugs” tend to have limited therapeutic options causing a significantly increased period of hospital treatment and thus much higher health care costs (Davies and Davies, 2010; Rubin et al., 1999).

Thus, with health systems all over the world being besieged by this multifaceted and complex problem of multidrug resistance, the need for new solutions is currently greater than ever before. The pathogenic virulence of these infections has been increasing as bacteria acquire new genes and methods of evading antibiotics. While there was an initial attempt to produce new antibiotics by large pharmaceutical companies to overcome the early rise of antibiotic resistance, there was a sharp decline in these developments with only two new classes of antibiotics being developed between the years 2000 and 2010 (Figure 2.2). This is owed to the incredible rate at which bacteria developed resistance against the new classes of antibiotics.

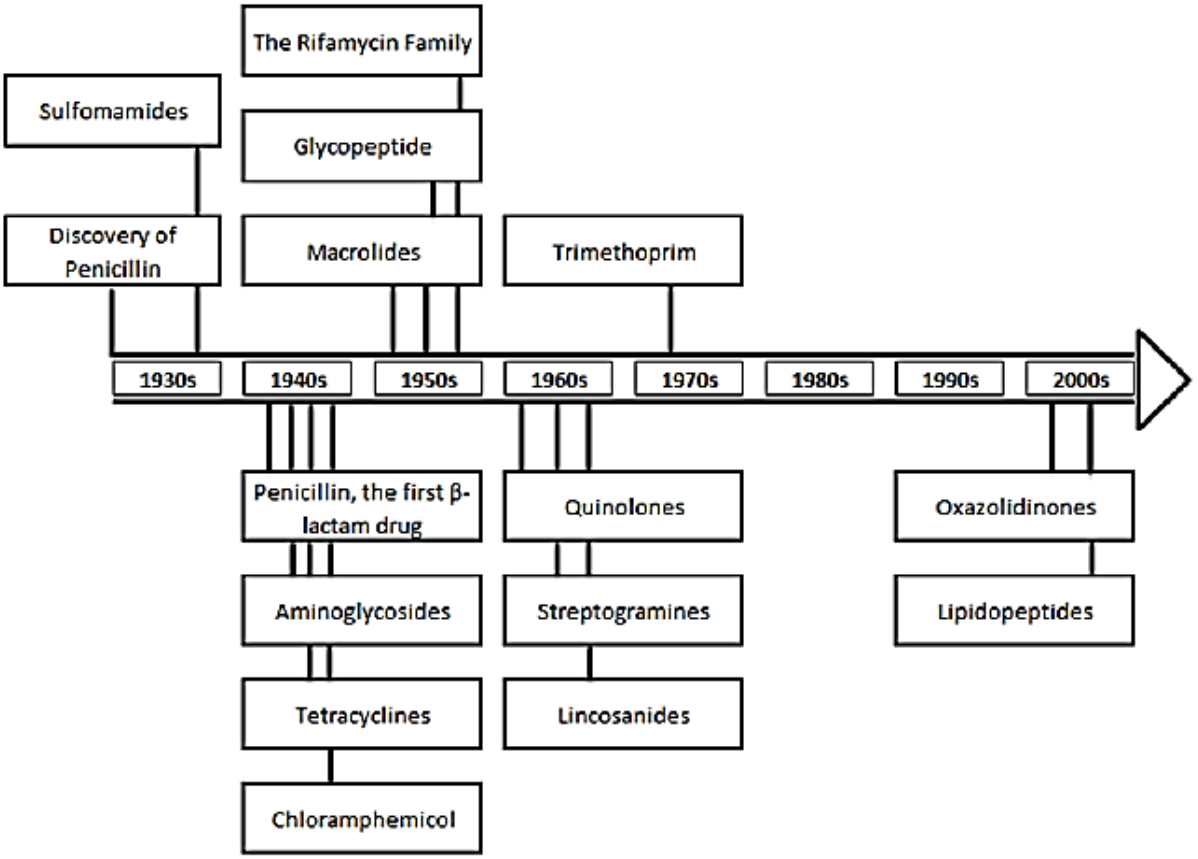


Figure 2.2 Timeline of new antibiotic classes. The timeline illustrates when the major antibiotics were developed during the past 70 years. During the 1950's new antibiotics peaked, this is known as the golden era, this is when most of the antibiotics that we used today were discovered. The lean years followed from about 1965, this was a low point in antibiotic discovery and development. Many attempts were made to understand and improve the use of antibiotics by pharmacological, biochemical, and selective target approaches, few were successful (Dowling et al., 2013).

Thus, it seems rather paradoxical that the leading pharmaceutical efforts of our generation have somehow lost interest in formulating solutions to what could very well be the greatest threat to public health in the history of modern medicine (Dowling et al., 2013). The reason that the development of new antibiotics has decreased sharply over the years (Figure 2.3) is probably owed to the massive costs associated with the development of new antibiotics. While Paul Ehlrich's method of wide screening different compounds was revolutionary in discovering the cure for syphilis (Bosch and Rosich, 2008), the same methods are not as practical in the modern clinical setting. The long and expensive development requires a long-term usage of the drug to cover the costs of research and development. However, bacteria developed resistance so rapidly that many of the antibiotics developed over years can see traces of resistance developing in a matter of weeks after they are released (Davies, 2012).

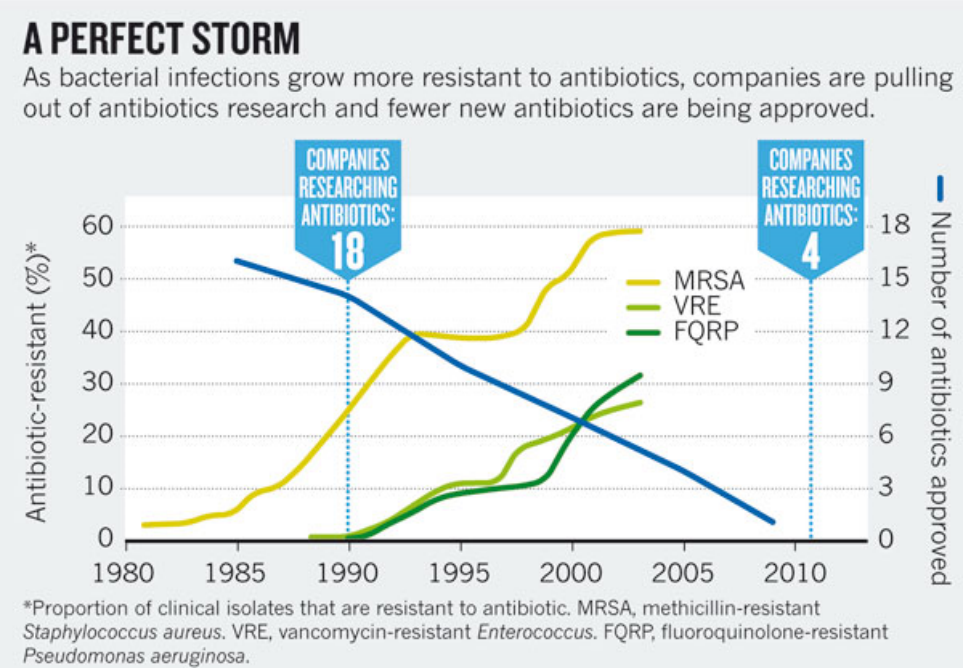


Figure 2.3 The decrease in new antibiotics compared to the increase in common antibiotic resistant pathogenic bacteria (Cooper and Shlaes, 2011).

Thus, the pharmaceutical industry's research and development efforts tend not to prioritize on developing antibiotics but tend to be more focused instead on long-term illness associated with aging populations which can prove profitable for many years, such as cancer, cardiovascular, psychoneurological and lifestyle-targeted therapies (Davies, 2012; Gibbons, 2008). Pharmaceutical companies tend to make more money when selling drugs

that one must take every single day for the rest of their life, rather than drugs like antibiotics that are only taken over a few weeks. The development of new antibiotics is not prioritized as it is less profitable in the long-term (Gibbons, 2008).

While pharmaceutical companies have been slow to fully appreciate the antibiotic crisis, the international and local authorities have been swift in recognising this crisis. While many reports, statistics, strategies and recommendations have been published by the WHO, the American Centre for Disease Control (CDC) and many other concerned individuals, it has shown little success in effectively controlling the spread of antibiotic resistance. Measures such as reducing the prescription and enforcing legislation to restrict antibiotic use has proved effective in reducing the incidence of antibiotic resistance infections. However, it was observed that while certain geographic areas could successfully implement such measures, antibiotic resistance disseminating from regions overusing antibiotics will invariably result in a reintroduction of antibiotic resistant pathogens and their mobile genetic determinants (Rogers et al., 2012).

It is recognized that a new approach different to those currently being widely utilized is necessary. The first consideration to finding alternative antimicrobial treatments is a source that has a long history of human practice and has been proven to be an effective source of antimicrobial chemotherapeutics. This source could be natural products, primarily plants (Gibbons, 2008).

2.2.2 Using plants in treating antibiotic resistance

Antibiotic resistance has been found to be a complex process incorporating numerous biochemical reactions, bacterial cellular organelle and the use of community interactions amongst pathogens in mutual assistance. It would thus seem ideal to state that an effective solution for confronting this complex ability would need to contain a certain level of biochemical complexity that can match, or at the very least compete with the intricate systems of antibiotic resistance (Brötz-Oesterhelt and Brunner, 2008). The chemical complexity often contained in nature is known to proceed well beyond the ability of human scientific mimicry. We simply cannot compete with nature and its chemical complexities and biochemical modes of action. Thus realistically and practically speaking, in order to

confront a complex biochemical challenge from nature, one needs to resort to a complex biochemical solution from nature (Abreu et al., 2012; Gibbons, 2008; Hemaiswarya et al., 2008).

The antibacterial properties of naturally occurring chemical compounds in plants have shown to be immensely versatile in nature and it is believed that these complex antimicrobial peptides and secondary metabolites may be the "magic bullet" that we so desperately need to solve the antibiotic resistance crisis in our age (Dowling et al., 2013).

2.2.3 Antimicrobial properties in plant extracts

In the 21st century, there has been a significant increase in the number of studies that have been carried out in searching for antimicrobial activity in plant products. There has been a particular focus on herbs and spices according to Hayek et al. (2013). It has been reported by Tajkarimi et al. (2010) that there were over 1340 plants that have been determined to have antimicrobial activities, and over 30,000 antimicrobial compounds have been isolated from these plants.

The reason for plants needing to produce effective antimicrobials is owed to their production of a large variety of secondary metabolites renowned for their therapeutic capabilities and are found in edible, medicinal and herbal plants as well as their derived essential oils (EOs) (Hayek et al., 2013). Gibbons (2008) notes three important reasons for plants being an effective source of antimicrobials:

i) Plants produce complex antibacterial compounds for their own chemical defence against soil-based bacteria.

ii) There are countless examples of plants being used in history for treating many ailments including pathogenic diseases, thus there is a rich ethnobotanical repository of information that could be readily used to identify ideal candidates for prospective treatments.

iii) The extensive functional groups and high chemical chirality in plants reflect a prominent chemical diversity that bacterial antibiotic resistance may not have historically been exposed to. This could mean developing resistance against these systems (Gibbons, 2008).

Antimicrobial compounds in plant materials have been found to be common in herbs and spices (e.g., rosemary, sage, basil, oregano, thyme, marjoram, cardamom, and clove) as well as in fruits and vegetables (e.g., guava, pepper, cabbage, garlic, and onion) and finally in seeds and leaves (e.g., grape seeds, caraway, fennel, parsley, and olive leaves) (Álvarez-Martínez et al., 2021; Tajkarimi et al., 2010).

The antimicrobial efficacy of the active antimicrobial plant compounds is largely dependent on their chemical structure and concentration. Secondary metabolites tend to be antimicrobial as indeed the antimicrobial phytochemicals isolated from most plants with antimicrobial effect including saponin, flavonoids, thiosulfinates, glucosinolates, phenolics, and organic acids. However, the main components in plants with the best antimicrobial activity are usually phenolic compounds such as isoflavonoids but also terpenes, aliphatic alcohols, aldehydes, ketones and acids (Hayek et al., 2013; Tiwari et al., 2009).

Though plant antimicrobial compounds generally have shown to have a much greater inhibition effect against Gram-positive than Gram-negative bacteria, it was observed that a small number of non-phenolic plant compounds found in essential oils such as allyl isothiocyanate and garlic oil are more effective or highly effective against Gram-negative bacteria. Thiosulfinates were noted to have a strong antimicrobial effect against Gram-negative bacteria in particular (Hayek et al., 2013). This proves the point postulated by Gibbons (2008) that the richer chemical diversity of plants gives them a greater reach as natural antimicrobial compounds.

As noted, plants are an indispensable solution in confronting pathogenic bacteria. While antibiotics have been growing less effective, it has been noted that amoxicillin, a moderate-spectrum, bacteriolytic β-lactam antibiotic, was considered to be the most prescribed antibiotic. In the 2000s, following the decline in antibiotic development, the only antibiotic that showed up on the top 20 prescription drugs was amoxicillin-clavulanic

acid, a combinational treatment featuring a β-Lactam antibiotic coupled with a β-lactamase inhibitor to preserve its effectiveness against antibiotic resistant bacteria. This shows a possible lead in using natural compounds in enhancing the activity of conventional antibiotics that were previously rendered ineffective (Dowling et al., 2013; Villa, 2012).

2.2.4 Synergism between antibiotics and antimicrobial plant products

The synergism between antibiotics and plant compounds has been well studied over the years. Plants produce a diverse group of antibiotics that have been observed to be highly effective against the mechanisms of antibiotic resistance. This was discussed in detail by Abreu et al. (2012) who noted that many antimicrobial phytochemicals are what they termed as resistance modifying agents (RMA). In their review, Abreu et al. (2012) discussed 40 such RMA which were found to be effective against a diverse number of antibiotic resistance mechanisms. These included phytochemicals able to inhibit different types of efflux pumps, inhibit the expression of PBP2a, a mutant to the antibiotic target PBP. In addition, Abreu et al. (2012) discussed a number of therapeutic plants whose extracts had been observed to enhance or restore the activity of antibiotics against antibiotic resistant bacteria (Abreu et al., 2012).

Some of the examples of plants discussed by Abreu et al. (2012) such as the synthesis of compounds that could synergistically treat antibiotic resistance mechanisms are common among spices and herbs. These include thymol and carvavrol, two essential oils from *Thymus vulgaris* (or thyme) which previous studies have shown to break down Gram-negative bacterial outer membranes thus increasing antibiotic penetration. Piperine which is found in *Piper nigrum* (or black pepper) increased the efficiency of the antibiotic ciprofloxacin against methicillin resistant *S. aureus.*

Figure 2.4 shows different antibiotic resistance mechanisms and how certain secondary plant metabolites can work directly against these mechanisms and restore antibiotic activity.

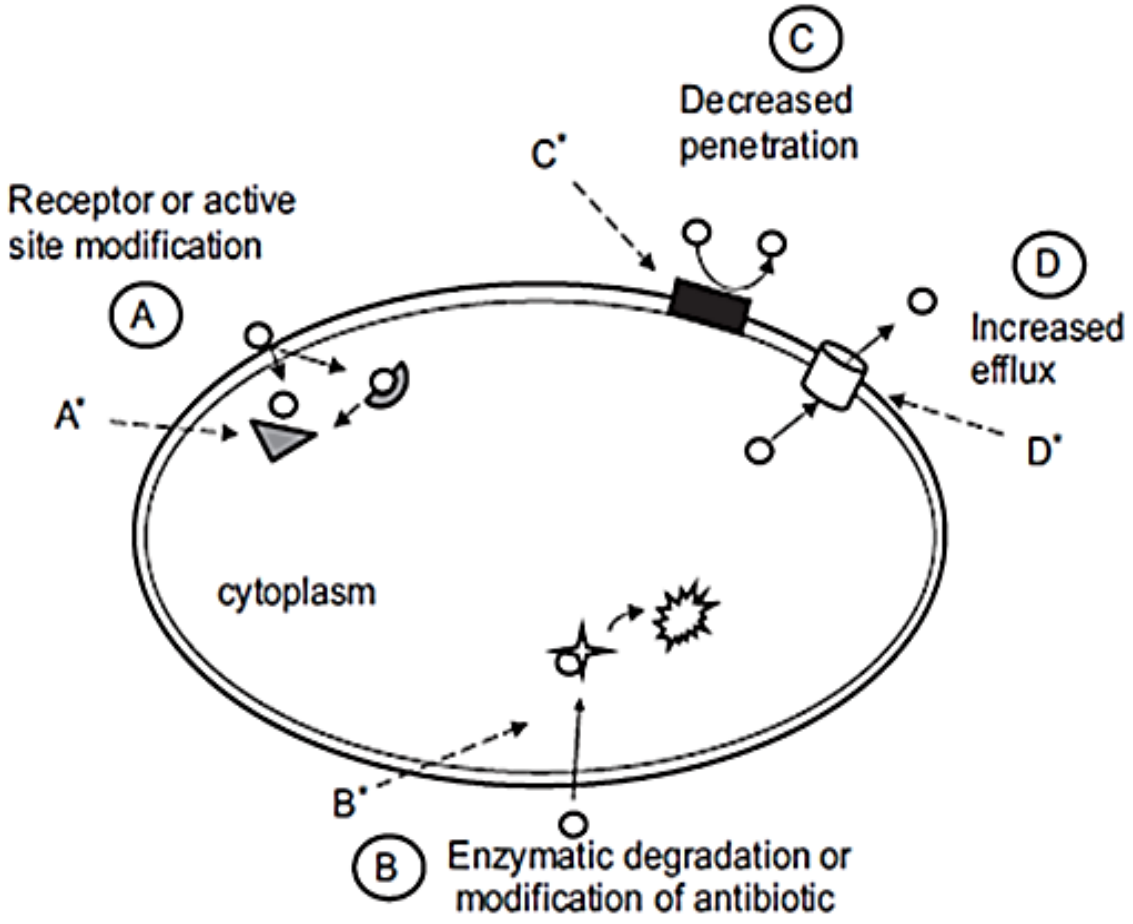


Figure 2.4: Plant secondary metabolites as modifiers of multidrug resistance mechanisms. - O- antibiotic drug, - receptor, - modified receptor, -efflux pump, enzyme, -degradation of the drug. (A*) corilagin, tellimagrandin I, diterpene 416 and compound P inhibits PBP 2a, a modified receptor; (B*) EGCg inhibits the β-lactamase; (C*) thymol, carvacrol, gallic acid increases the outer membrane permeability; and (D*) EGCg, 5'-methoxyhydnocarpin, reserpine, carnosic acid and isopimarane derivatives inhibit the efflux pumps (Hemaiswarya et al., 2008)

A large variety of plant antibacterial compounds (including phytoalexins) depend on synergistic mechanisms when secreted in defence of the plant to neutralize bacterial threats. Most of these terpenoids, glycosteroids, flavanoids etc. individually contain very limited antimicrobial capabilities. However, when secreted in combination with one another (or a myriad of other phytochemicals), they are able to increase in antibacterial strength and versatility to neutralize a wide variety of microbial invasions. This was discussed in detail by Hemaiswarya et al. (2008) and is shown in Table 2.3. Figure 2.4

(Scheme D) shows two efflux pump inhibitors berberine and 5'-methoxyhydnocarpin, both produced by the barberry plants. Berberine is a hydrophobic alkaloid that intercalates into DNA from whence it is immediately expelled by the microbe's efflux pumps making it grossly ineffective as an antibacterial. When combined with 5'-methoxyhydnocarpin which blocks the efflux pump, the concentration of the berberine in the bacterial cell is increased thus allowing it to enter the cell and regain its antibacterial activity. Indeed, it was reported that coupling berberine to an effective efflux pump inhibitor, INF_{50}, restored the highly effective antibiotic properties of berberine (Ball et al., 2006). There are many other examples of synergistic antibacterial properties that specifically work against the mechanisms of antibiotic resistance as shown in Table 2.3 which indicate that synergism is a tried and tested method of overcoming multidrug resistant bacteria.

The remarkable synergism between plant compounds and antibiotics against antibiotic resistant bacteria is by no means a serendipitous coincidence. It stems from the fact that plant antimicrobials might be designed to work against bacteria not as individual compounds but in combination with other plant antimicrobial agents. Considering that plants aren't besieged by bacterial infection or succumbing to antibiotic resistant infections shows that the systems and strategies they employ are actually working against a problem that modern medicine and science has failed to solve (Hemaiswarya et al., 2008).

What should be noted is that most of these synergism analyses were carried out *in vitro*. All the 40 phytochemicals Abreu et al. (2012) reviewed in their paper were found to potentiate a list of conventional antibiotics which was attributed to their activity against the antibiotic bacteria's resistance mechanisms. Gibbons (2008) also reviewed phytochemicals that have been shown to have activity against antibiotic resistant bacteria. The 28 phytochemicals reviewed by Gibbons (2008) were also carried out *in vitro,* although owing to their toxicity a number of antibacterial phytochemicals were better suited for external use in ointments and creams rather than internal use.

In Table 2.3 Hemaiswarya et al. (2008) were able to identify various natural products that could work effectively with specific antibiotics to restore their effectiveness in treating antibiotic resistance. For example, corilagin and tellimagrandin I found in *Punica granatum*

and *Eucalyptus globus* respectively were both found to be effective against modified Penicillin Binding Proteins (PBPs). Epigallocatechin gallate (EGCg) was found to be a beta-lactamase inhibitor while thymol from the thyme herb was found to increase cell membrane permeability thereby increasing antibiotic penetration. And finally, efflux pumps were inhibited by reserpine and 5'-methoxychydnocarpin (Hemaiswarya et al., 2008). This could present a future model for overcoming antibiotic resistance by creating a repertoire of natural products effective against specific antibiotic resistance mechanisms and the antibiotics they are compatible with, as can be seen in Table 2.3.

Table 2.3 Synergism between natural products and antibiotics against bacterial infection (Hemaiswarya et al., 2008).

Natural product	Antibiotics	Microorganisms	Mechanism of action
Carnosic acid	Tetracycline	Tet (K) possessing strains	Inhibit the MDR pumps, Tet (K) and Msr (A)
Carnosol	Erythromycin	Msr (A)	
Epigallocatechin-gallate (EGCg)	Ampicillin/sulbactam	MSSA β-lactamase producing *S. aureus*	Inhibits β-lactamase
EGCg	Penicillin	Penicillinase producing *S. aureus*	Inhibits penicillinase
	Ampicillin		
EGCg	Carbapenems	MRSA	–
EGCg	β-Lactam	MSSA, MRSA	EGCg directly binds to the peptidoglycan and inhibits cell wall
EGCg	Tetracycline	*S. aureus* with Tet (K) MDR pump	Blocks MDR efflux pumps
Tea catechin	Oxacillin	MRSA	–
Totatrol	Methicillin	MSSA, MRSA	PBP 2a production
Berberine (*Berberis* plant)	5′-Methoxyhydnocarpin	Nor (A) mutant	Inhibits Nor (A) MDR pump
Green tea extract	Levofloxacin	*Escherichia coli* 0157 in gnotobiotic mouse model	–
Craneberry juice extract Blueberry, Grape seed and oregano extract	–	*Helicobacter pylori*	–
Oregano and cranberry extract	Lactic acid	*Vibrio parahemolyticus*	–
Isoflavone Bidwillon B from *Erythrina variegata*	Mupirocin	*MRSA*	Bidwillon B and mupirocin inhibited the incorporation of thymidine, uridine, glucose and isoleucine
α-Mangostin	Vancomycin	*MRSA and Vancomycin enterococci*	–
	Gentamycin		
Aqueous crude khat extracts	Tetracycline	*Streptococcus sanguis, Fusobacterium*	–

Corilagin from *Arctostaphylos uva-ursi*	β-Lactams such as oxacillin , cefmetazole	MRSA	Inhibits PBP2a production or activity
Baicalin	β-Lactam antibiotics	MRSA	Inhibits β-lactamase
Tellimagrandin I from rose red tree,	β-Lactams	MRSA	–
Rugosin B from rose red tree	β-Lactams	MRSA	–
Diterpenes from *Lycopus europaeus*	Tetracycline	*S. aureus* possessing Tet (K), Msr (A) MDR pumps	Blocks MDR pumps
	Erythromycin		
A penta-substituted pyridine from *Jatropha elliptica*	Erythromycin	*S. aureus* possessing Tet (K), Nor (A) MDR pumps	Blocks MDR pumps
	Ciprofloxacin		
Pomegranate extract	chloramphenicol, gentamicin, ampicillin, tetracycline, and oxacillin	*MRSA*	Blocks Nor (A) pump
		MSSA	
Myricetin	Amoxicillin/clavulanate, ampicillin/sulbactam and cefoxitin	Extended-spectrum -lactamases (ESBL) producing *K. pneumoniae*	–
Isopimaric acid from *Pinus nigra*	Reserpine	MRSA	Blocks Nor (A) pump
Totarol, ferulenol (from *Ferula communis*) and plumbagin (from *Plumbago zeylanica*)	Isonicotinic acid hydrazide (INH)	*Mycobacterium intracellulare*, *M. smegmatis*, *M. xenopei* and *M. chelonei*	–
Erybraedin A or eryzerin C isolated from the roots of *Erythrina zeyheri*,	Vancomycin	Vancomycin-resistant enterococci (VRE) and MRSA	–
Butylated hydroxyanisole, (BHA) green tea		*S. mutans*, non-susceptible *E. coli* and *C. albicans*	–
Sophoraflavanone G	Vancomycin hydrochloride, fosfomycin, methicillin, cefzonam, gentamicin, minocycline and levofloxacin	MRSA	–

Essential oil – 1,8 cineol, linalool, alpha-terpineol and terpinen-4-ol from *Melaleuca leucodendron* and oil from *Ocimum gratissimum*	Antibiotics	Bacterial species	–
Novoimanin from *Hypericum perforatum* L.	Ampicillin, kanamycin, fusidic acid and rifocin	*Staphlococcus aureus* 209	–

The period from antibiotic discovery to antibiotic clinical trials is often long and expensive. While there is a need to discover effective antibacterial treatments against antibiotic resistance, there is a great need for more methods of analysing and attaining more data for these trials to shorten the path to effective clinical trials and drug development in order to get effective treatments out to hospitals and patients as soon as possible.

The use of plants' secondary metabolites in combination with antibiotics in order to potentiate their antibacterial activity (Table 2.3) is a promising development and has been studied with the various antibiotic resistance mechanisms having been identified (Hemaiswarya et al., 2008). Kongkham et al. (2020) extensively reviewed this solution, along with the use of plant secondary metabolites (PSMs) as antibiotics and observed that the trend of increasing diversity in antibiotic resistance mechanisms combined by the increase in antibiotic classes has resulted in a narrowing of the antibiotic pipeline. New and stronger antibiotics often cause bacteria to become more antibiotic resistant due to the increased stresses. They also observed that plant secondary metabolites combined with antibiotics could be an effective way to reverse the trend of a narrowing antibiotic pipeline and instead to re-widen the pipeline (Figure 2.5). However, combining PSMs with antibiotics will not solve the problem of antibiotic resistance completely. The full potential of this synergistic approach between PSMs and antibiotics is yet to be fully realized due to a limited knowledge of plant secondary metabolite structures and their activity against bacteria (Kongkham et al., 2020).

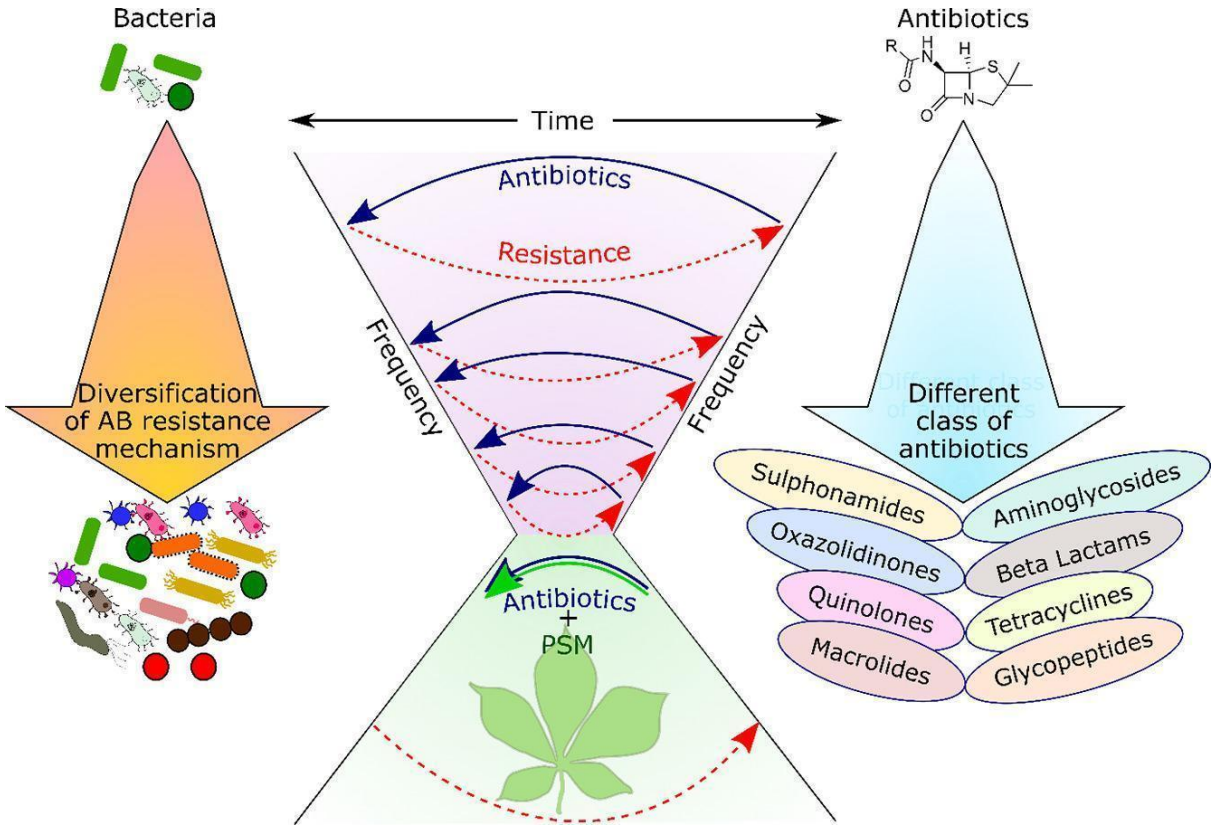


Figure 2.5: Interaction of antibiotic and bacteria over time results in development of new resistant bacterial strains having different mechanisms of resistance. This happens at an increasing frequency narrowing the antibiotic pipeline (centre). The use of PSMs along with antibiotics can help in countering this never-ending threat and thus widening the antibiotic pipeline add reference and full-stop at end (Kongkham et al., 2020).

Chapter 3: Comparison of Metabolites of Antibiotic Susceptible and Resistant *Staphylococcus xylosus* strains

3.1 Introduction

An assessment of the current antibiotic resistant crisis described in Chapter 2 showed that it is a complex phenomenon that began well before humans discovered antibiotics and may have existed in bacteria for millions of years. The acquisition of resistance, especially among clinical strains, came after a process through which there was a change in the bacteria's environment with an increased use of antibiotics and disinfectants. Resistance to antibiotics emerged as either an active or a passive process which reflected a change in bacterial metabolism. In order to explore this change, a comparison of the susceptible wild-type strain and a consequently resistant strain of *Staphylococcus xylosus* was carried out.

The first objective was to select an antibiotic susceptible strain of bacterial species and then create a new antibiotic resistant strain from this antibiotic susceptible wild-type strain. This was then followed by a comparison of their metabolic profiles by NMR-based metabolomics.

3.2 Experimental design and methods

The two objectives of this first part of the study were investigated by means of four main experimental procedures:

i) Selecting a suitable bacterium culture that is antibiotic susceptible.
ii) Producing an antibiotic resistant strain from this antibiotic susceptible culture.
iii) Extracting the intracellular metabolites of both bacterial cultures.
iv) Analysing and comparing these intracellular metabolites by means of ^{1}H-NMR spectrometry and multivariate analyses.

These four steps encompassed the layout of the first experiments with the details of each step being described in the experimental procedure below.

3.2.1 Selecting a suitable bacterial culture that is antibiotic susceptible

Considering the health hazards of working with antibiotic resistant pathogens and the lack of sufficiently adequate biosafety protection against most pathogenic bacteria in the laboratory where the study was to be carried out, the bacterial species to be used was to be a non-pathogenic one. This was the first and most important condition in the selection of a necessary bacterial culture if the experiment was to be completed safely.

It was also ideal that the bacterial culture being used was versatile in growth i.e., able to grow effectively under the most basic conditions and not requiring special growing conditions or specialised medium.

In order for the study to be relevant to the problem that it was designed to confront, there had to be a link albeit a certain degree of similarity between the bacterial culture chosen and previously identified infectious antibiotic-resistant pathogens.

The Gram-positive, coagulase-negative bacterium culture, *Staphylococcus xylosus* was selected based on these criteria when designing the experiment. *S. xylosus* is commonly isolated from the mammalian skin microflora as well as food products such as milk, cheese and meats. *S. xylosus* has industrial applications in food production as a starter culture combined with lactic acid bacteria during sausage and cheese production (Dordet-Frisoni et al., 2007). Thus, the culturing could be carried out on standard media using the standard available incubators of the laboratory. In comparing the traits that distinguish various pathogenic staphylococci, Rosenstein and Götz (2013) stated that *S. xylosus* was a non-pathogenic staphylococcus.

This was confirmed by Dordet-Frisoni et al. (2007) that the bacteria can be defined as virtually non-pathogenic. There have been cases of *S. xylosus* causing acute pyelonephritis and secondary root canal infection in humans (Siqueira et al., 2002) however, these infections occurred alongside other primary staphylococcal invasions and were more opportunistic than virulent. Furthermore, the reference culture for *S. xylosus* (ATCC® 29971™) has a recommended biosafety of level 2. In terms of antibiotic resistance, the reference culture for *S. xylosus* is resistant against novobiocin but mostly susceptible to other antibiotics (Rosenstein and Götz, 2013).

A significant link between methicillin-resistant *Staphylococcus aureus* (MRSA) and *S. xylosus* was shown with the identification of the *mecC* gene present in certain strains of MRSA. MRSA typically expresses the *mecA* gene, which is responsible for conferring resistance to β-lactam antibiotics by altering the bacterial cell's PBP protein targets. *S. xylosus* strains have been identified which contain a *mecC* variant, which is very similar to the same gene found more recently in some variant strains of MRSA. The *mecC* carrying MRSA was just as virulent as other MRSA. The only difference between mecC carrying MRSA and the more common mecA carrying MRSA was that the mecC carrying variant was harder to detect during analysis, since MRSA detection is often carried out on *mecA* (Paterson et al., 2014).

Another clinical relevance of *S. xylosus* was that it was also very similar to other coagulase negative staphylococci like nosocomial staphylococcal pathogens *S. epidermis* and *S. saprophyticus* (Assad et al., 2016).

3.2.2 Producing an antibiotic resistant strain from the antibiotic susceptible culture

Antibiotic susceptible bacteria develop mutations and resistance to antibiotics when exposed to stress conditions such as starvation, antibiotic stress, pathogenic stress (Martinez and Baquero, 2000). Thus, it was expected that placing the *S. xylosus* strain under such stresses may develop an antibiotic resistant strain. In the absence of viable antibiotics, it was decided to use starvation of the culture as a stress factor to incur antibiotic resistance. The most basic way to achieve this would be allowing the bacterial culture to grow in a culture medium for a number of days. This has been shown to increase the rate of mutation in bacteria cultures, particularly noted in the fusion of *araB-lacZ* genes under starvation of glucose indicating mutation by starvation stress (Maenhaut-Michel and Shapiro, 1994).

Therefore, the initially antibiotic susceptible strain could be considered as the wild-type while the created resistant strain as the mutant (as it probably underwent mutation).

3.2.3 Extracting the intracellular metabolites of the bacteria cultures

The extraction of the bacterial metabolites needed to be carried out in a manner that preserved the structural integrity of the compounds so that they could be analysed. It is

vital that any possible factors that can change the nature of the metabolites were restricted, such that the results obtained are limited to only those biologically relevant activities that were assessed. These protocols coupled with multiple replicates should give an accurate result of the metabolome. Considering that this would be a comparative analysis, it was also important that a great deal of care was taken to treat each sample exactly the same way so as to minimize error and ensure consistency in results (Broadhurst and Kell, 2006).

3.2.4 Analysing the intracellular metabolites by means of ^{1}H-NMR spectrometry

Nuclear magnetic resonance (NMR) spectrometry has been used in studying the metabolites of a wide range of biological systems (Simmler et al., 2014). It is a vital tool when carrying out systems biology analyses and can present the quantitative and qualitative measurement of metabolites from extracts. In the case of this experiment, that includes cellular processes for antibiotic resistance. NMR-based metabolomic studies include the following general steps: cell growth and harvesting, metabolite extraction, NMR data collection and analysis, multivariate statistical analysis, metabolite identification and quantification (Cuperlovic-Culf et al., 2010). Often ^{1}H-NMR spectrometry is used in multivariate analysis such as principal component analysis (PCA) or orthogonal projection to latent structures discriminant analysis (OPLS-DA) (Halouska et al., 2013).

3.3 Materials and Methods

3.3.1 Culturing the bacteria

The selected bacterium*, S. xylosus* was obtained as a reference culture ATCC 29971 (or DSM 20266) (Microbiologics, USA). The culture cue tip was streaked on to 6 Standard I nutrient agar-containing Petri dishes (Biolab diagnostics) to attain isolated colonies. The plates were then incubated in an incubator (Labotech, South Africa) at 37 °C.

These were then retrieved from the incubator after 48 hours and pure white colonies characteristic of *S. xylosus* (Kloos and Schleifer, 1986) were observed to have been growing

on the agar. These cultured plates contained the original wild-type (antibiotic susceptible bacteria) and were then placed in a refrigerator at about 3-5 °C for a period of one week.

3.3.2. Attaining the antibiotic resistant mutant from the susceptible strain

Before an antibiotic resistant mutant could be produced, the previously cultured wild type was tested for antibiotic resistance.

A pure colony of the wild-type mutant was scraped off the agar plate by a flamed loop and added to 100 ml of Standard I nutrient broth in duplicate in 2 250 ml conical flasks. The two broths were incubated in an incubator at 37 °C for 48 hours without shaking. 100 µl of each of these broth samples (with bacteria) was pipetted onto a fresh standard agar plate and streaked over the agar. The two broth cultures were put back into the incubator.

To determine the sensitivity of the culture to antibiotics, the Kirby-Bauer disc diffusion method was employed (Biemer, 1973). Three small susceptibility disks containing 10 µg of penicillin (Biolab, Hungary) were placed gently on each of the agar plates equidistant from one another. The plates were incubated in an incubator for 48 hours and tested for susceptibility to penicillin. Of the two broth cultures, one was left in the incubator for 2 weeks while the second was left in the incubator for 4 weeks.

After incubation, the 2 week and 4-week-old broth cultures were tested for antibiotic susceptibility and the 4-week-old culture was found to be completely antibiotic resistant when grown on standard agar Petri dishes with 50 µg penicillin and methicillin (Biotech, Hungary) discs.

3.3.3. Extracting the intracellular metabolites of the bacterial cultures

After having confirmed the wild-type susceptibility and mutant resistance to antibiotics, 2 stock cultures of each strain were re-cultured in 100 ml of tryptic soy broth (Merck, Germany) and incubated for 48 hours. All the replicates to be prepared for metabolomic analyses were cultured from these. These cultures were kept in a walk-in fridge with a temperature of between 3 – 5 °C.

Six 300 ml conical flasks were filled with 120 ml of autoclaved tryptic soy broth. Eight of these flasks were inoculated with 100 µl from the antibiotic resistant stock each and the other 8 were inoculated with 100 µl from the antibiotic susceptible stock. All 16 of the inoculated cultures as well as 2 controls were incubated in a rotating incubator set to 120 rpm at 36-37 °C for 48 hours. After 48 hours, all the samples were moved to a refrigerator and stored for one day at 3-5 °C.

20 ml of broth was collected in separate sterilised 100 ml conical flasks from every one of the 16 conical flasks for further culture resistance analysis. The remaining 100 ml of tryptic soy broth was centrifuged at about 2 150 rpm in a Genevac (EZ-2 series, SP Scientific, England) (Figure 3.1) for a full 2-hour cycle to obtain separation between the bacteria and the broth. The broth (without bacteria) was carefully decanted in separate conical flasks for every replicate.

The precipitated bacterial pellets were quenched by adding 100 ml of analytical grade cold methanol (Sigma-Aldrich, South Africa) at -20 °C to terminate biochemical activity.

The bacteria were then ultrasonicated (Model AC-200H, MRC) for 15 minutes at a frequency of 40 kHz in order to break the cells and collect the cellular metabolites in the methanol. The methanol solution was then evaporated in a Genevac for 4 hours at 23 °C.

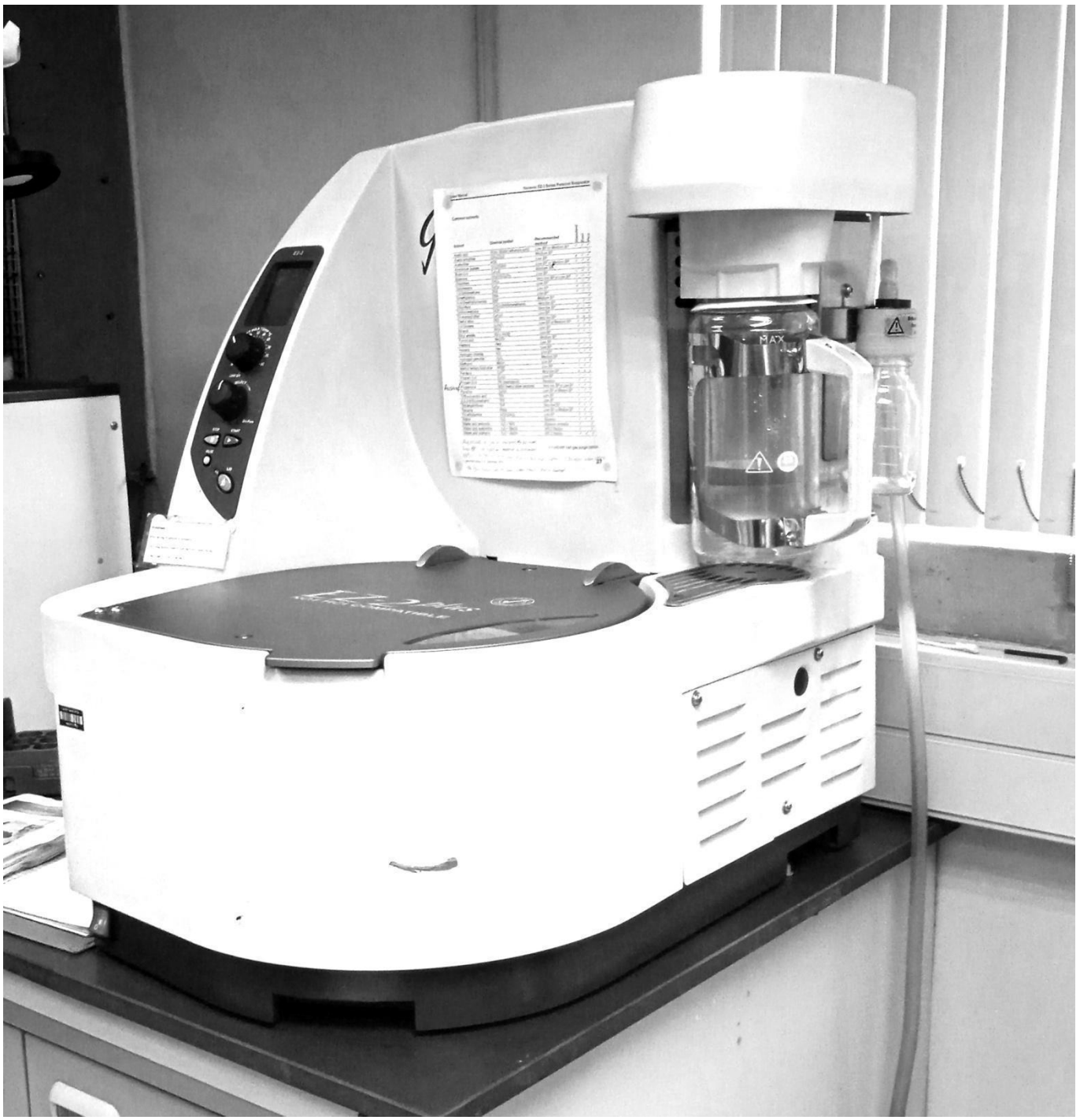


Figure 3.1 The Genevac (EZ-2 series, SP Scientific, England) used to separate the broth from bacterial supernatant as well as drying the methanolic extracts

Afterwards, the solution was quantitatively transferred to a weighed polytope tube and allowed to dry completely.

3.3.4. Nuclear magnetic resonance (NMR) and metabolomical analysis of bacterial metabolites

All dried extracts were dissolved in D_2O with the appropriate volumes so that the final concentration was 10 mg/700 µl.

Before spectra acquisition, the magnetic field of a 200 MHz Varian NMR spectrometer (Figure 3.2) was shimmed for D_2O and then scanned 256 times to obtain the spectral data. The spectra were processed (baseline corrected, phased and referenced) with MestReNova version 10 (Mestrelab Research, Spain).

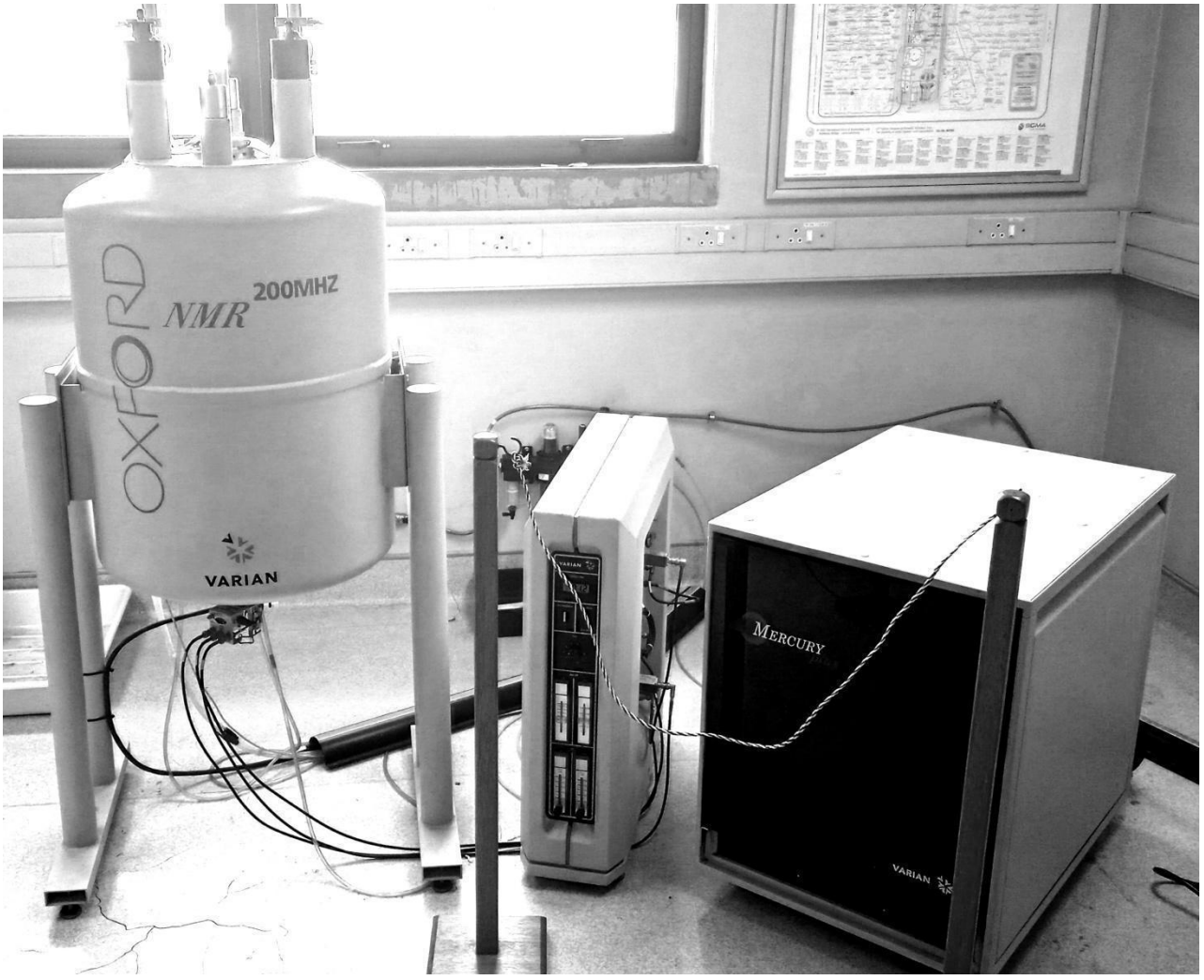


Figure 3.2 The 200 MHz Varian NMR spectrometer used for ^{1}H-NMR analyses

In the absence of an internal standard, the ^{1}H-NMR spectra were normalized with the water (solvent) peak at 4.81 ppm and set as the reference peak. The multivariate analysis was carried out on SIMCA version 14 (Umetrics, Sweden). All the ^{1}H-NMR spectra were uploaded to SIMCA where the program divided the entire length of each spectrum into small, equal regions of defined length called 'bins' or 'buckets' (0.04 ppm each). These bins were then analysed by principal component analysis (PCA) and each bin was compared to all the bins in the same region on all the other spectra. This method of analysis is particularly effective in comparing and grouping large sets of spectral data (Heyman and Meyer, 2012). The analysis was unsupervised, meaning that the data wasn't labelled as being from the antibiotic susceptible or resistant strain. The results were plotted on a PCA chart which determined how the different extracts would group based on their similarities and differences.

3.4 Results and Discussion

3.4.1 Susceptibility testing on disc

The first susceptibility test was to confirm that the wild-type (original) *S. xylosus* culture was antibiotic susceptible. The results in Figure 3.3 show that it was completely susceptible with no bacterial colonies growing within the antibiotic zone around all three penicillin discs.

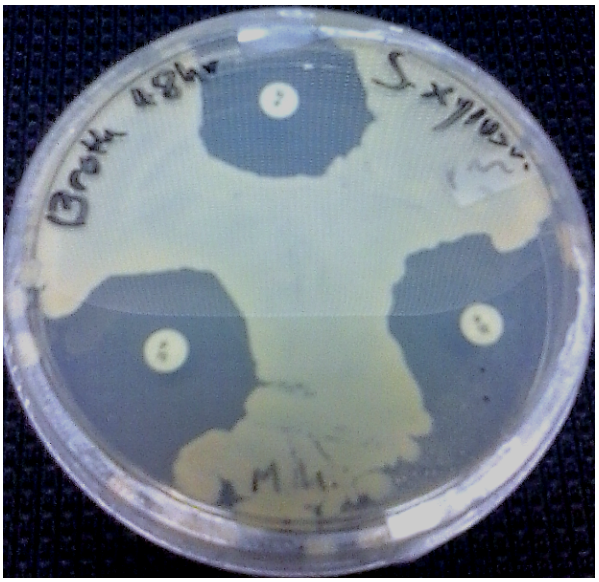


Figure 3.3: Kirby-Bauer Susceptibility test of wild type *S. xylosus* against penicillin on standard nutrient agar

Once this susceptibility was confirmed, two samples from the original (susceptible) culture were left in the incubator and the same growth medium for a number of weeks. The bacterial culture incubated in the same broth for 2 weeks seemed to grow around the antibiotics with a clear inhibition zone as expected. However, while there were clear zones of inhibition observed around the antibiotic regions, it was noted that a few colonies were growing inside the two zones of inhibition (Figure 3.4). This was not observed in the susceptibility test for the first wild-type culture (Figure 3.3).

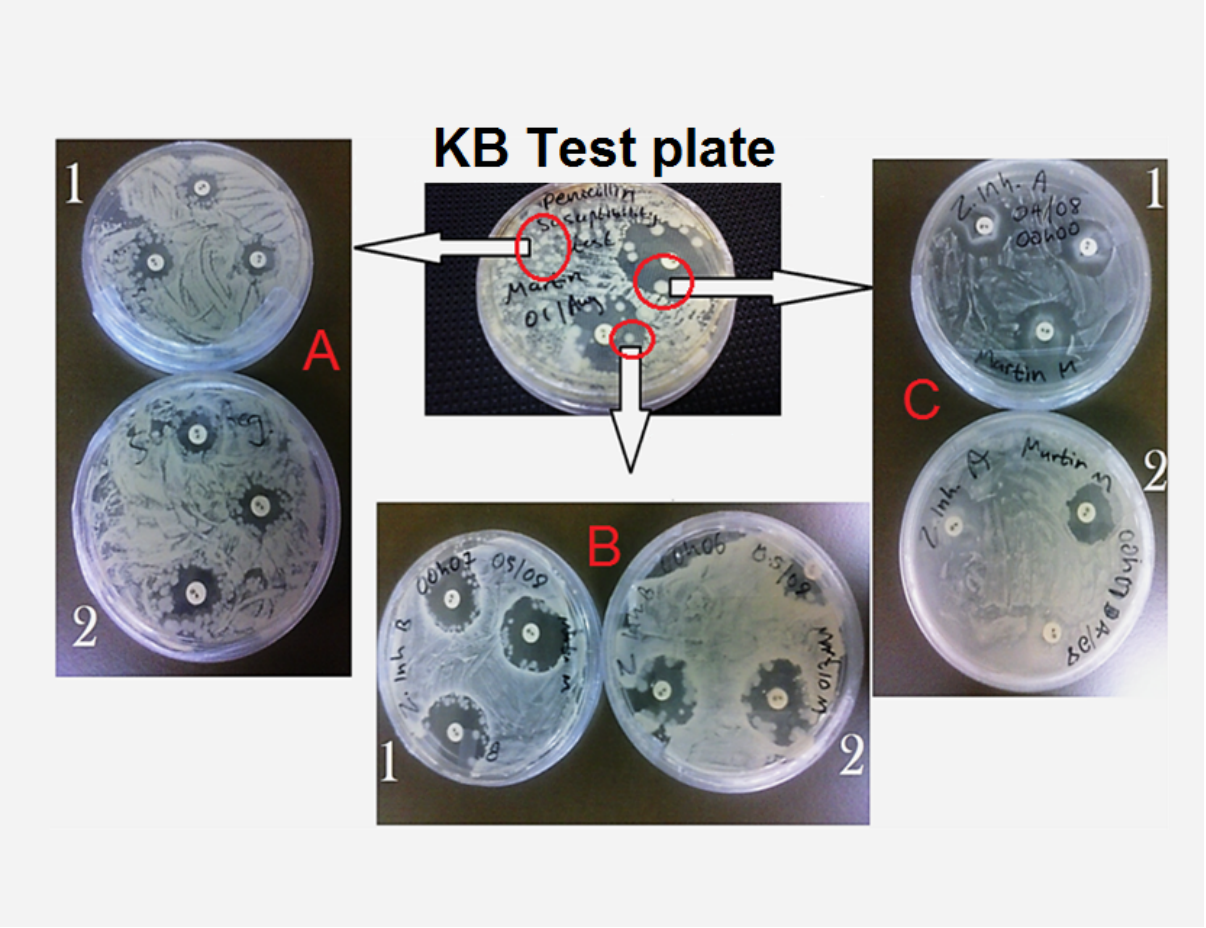


Figure 3.4 The Petri dish in the centre (KB Test plate) was a Kirby Bauer test carried out for penicillin by re-culturing from broth that had been inoculated with a pure *S. xylosus* culture and left in a non-swirling incubator for 2 weeks. Results showed that some of the re-cultured bacterial colonies were able to grow in the regions of inhibition. Some of these colonies (circled in red) were then re-cultured in duplicates. Duplicates A were cultured from a region with no antibiotic activity, duplicates B and C were taken from the colonies within the antibiotic region. All antibiotic discs shown contain the same antibiotic at the same concentration (50 µg penicillin). The replicates are numbered.

Considering that both susceptible and resistant agar plate cultures were streaked by the same method and incubated for the same period of time, the difference in antibiotic tolerance may have been a result of a change that the prolonged starvation incubation period may have elicited in the 2-week-old broth culture. The colonies growing in the antibiotic zones of inhibition were re-cultured for susceptibility testing (Figure 3.4 B and C). One colony from a region not containing antibiotics was also re-cultured for susceptibility testing. While it was observed that a zone of inhibition was initially formed around the antibiotic, there were a number of small colonies observed growing in the antibiotic region. This was observed particularly for Petri dishes A and B in Figure 3.4. It was also observed that the zones of inhibition for A were much smaller than the zones of inhibition in B. This was an interesting observation considering that B was taken from a region with antibiotic activity while A was taken from a region with no observable activity, and thus it would have been expected that B would have smaller zones of inhibition (due to exposure to antibiotics) while A was expected to have had larger zones of inhibition (due to a lack of exposure to antibiotics).

Following the observations made in the susceptibility tests using 50 µg penicillin antibiotic discs, the resistant bacteria cultures observed in Figure 3.4 (A, B and C) were re-cultured for methicillin and penicillin susceptibility tests. These tests were run over 4 days rather than the 2 days. This is because it was observed that the starved cultures had the propensity to develop more tolerance to antibiotics when left to grow on the medium for a longer period of time.

Similar trends were observed with the cultures incubated over 2 days, mainly a distinct circle forming the zone of inhibition around the antibiotic region and individual colonies growing inside the antibiotic zone of inhibition. The main difference between the results in Figure 3.4 (A and B) which had been exposed to some stresses and the cultures in Figure 3.1 which wasn't exposed to any significant stress was that the bacterial colonies that were exposed to more stress had a much smaller zone of inhibition.

When susceptibility tests were carried out with two different antibiotic discs (penicillin and methicillin) added to the same Petri dish (Figure 3.4), the same results were observed as

those of adding the same antibiotic to one plate. The agar cultures in Figures 3.4 did not show consistency in the bacterial lawn of growth with many of the cultures growing in a patchy fashion. It is likely that the bacterial colonies were forming in a patchy, disrupted pattern due to the production of biofilms. Planchon et al. (2006) determined that the *S. xylosus* species used in this study (DSM 20266) was able to produce biofilms. Biofilm formation causes cells to stick to a solid surface before they accumulate and aggregate to form clumps as the cells stick to each other by intracellular adhesion (Christensen et al., 1985).

Therefore, the zone of inhibition in which the bacteria were unable to grow on in Figure 3.4 was much smaller than those of Figure 3.3.

The sensitivity tests in Figures 3.1 and 3.4 showed some level of sensitivity to the antibiotics. This was observed by the defined circle forming around the antibiotic region and a change in the colony density within the antibiotic region. After observing the differences in the size of zone of inhibition without bacteria resulting from longer incubation periods, the bacteria were re-cultured in broth and incubated for a longer time to observe the differences in the zones of inhibition as well as bacterial sensitivity to the antibiotics. Figure 3.6 showed an equal response by the bacteria cultures to both methicillin and penicillin.

Figures 3.5 and 3.6 show bacteria re-cultured for antibiotic susceptibility tests were prepared by the same methods, except for the length of time that each culture was incubated in broth. The cultures in Figure 3.5 were incubated in the same broth for 14 days (2 weeks) before being re-cultured on the agar plates. The cultures in Figure 3.6 were incubated in broth the same for 28 days (4 weeks) before being re-cultured on the agar plates. Both duplicates were incubated for 24 hours. There were stark differences observed in the susceptibility to antibiotics.

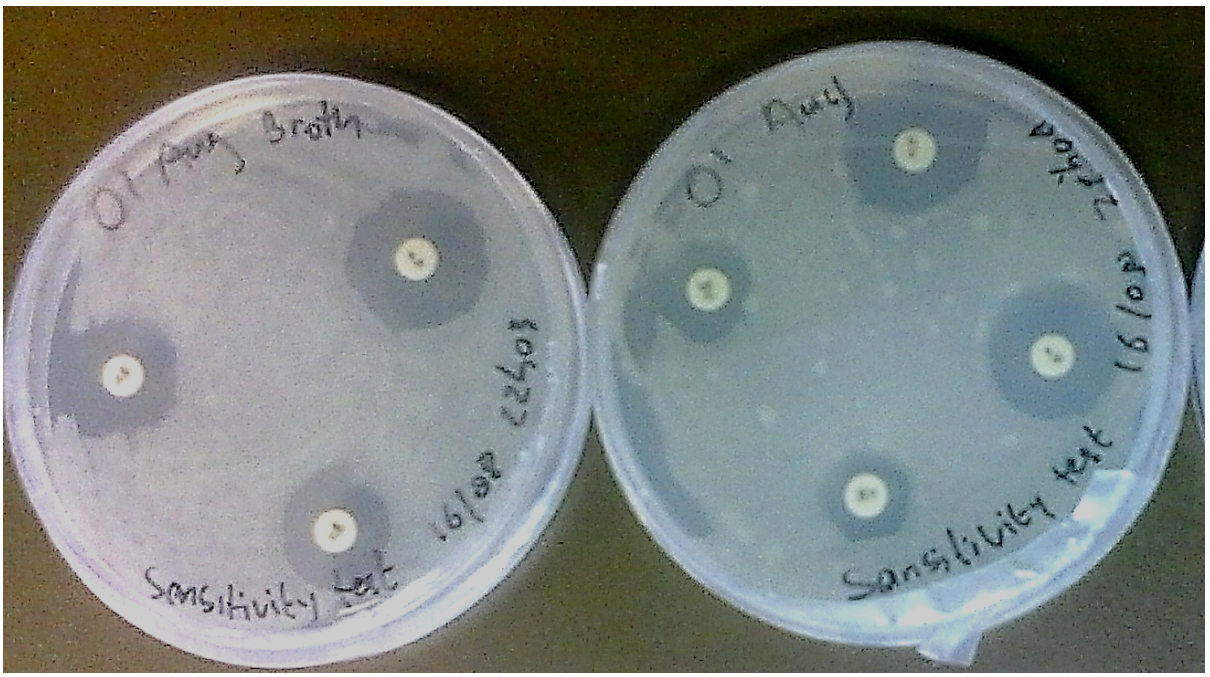


Figure 3.5 Susceptibility test for susceptible bacteria re-cultured from broth cultures incubated for 14 days. Red circles are penicillin discs while the yellow circles are methicillin discs.

Figure 3.6 Susceptibility test for resistant bacteria re-cultured from broth cultures incubated for 28 days. Red circles are penicillin discs while the yellow circles are methicillin discs.

The 2-week-old "starvation" cultures showed complete sensitivity to antibiotics with clearly defined zones of inhibition in which no cultures were observed to grow. On the other hand,

the 4-week-old “starvation” cultures showed complete insensitivity and resistance to the same antibiotics. This seemed to show that the period of prolonged incubation in the same broth (with limited resources) made the main difference between a full sensitivity to antibiotics versus a complete lack of sensitivity to antibiotics.

In terms of the bacterial lawn of growth for the results in Figure 3.5 and 3.6, it was noted that there was an improvement in the use of different streaking and incubating methods. The streaking for the cultures shown in Figures 3.5 and 3.6 was made using a glass “hockey stick” for spreading the culture onto the agar (Pye et al., 1995). It was much easier to obtain results for the susceptibility tests when a consistent bacterial lawn was established. Thus, the swirling incubator is most ideal when carrying out these tests as the formation of biofilms in the stationary cultures can disrupt the formation of a consistent bacterial growth lawn. Also, the “hockey stick” streaking method proved to be much better than using the wire loop for spreading the bacteria cultures on the agar.

As observed in these results, the bacterial culture which were initially susceptible to antibiotics had developed resistance by growing in limited nutrients or starvation stress. How was this antibiotic resistance achieved?

The mechanism for this lack of sensitivity to antibiotics may possibly be attributed to non-inherited antibiotic resistance. The phenomenon observed as the previously susceptible bacteria cultures were able to survive in antibiotic regions has been defined by McDermott (1958) as microbial persistence. McDermott (1958) further postulated that the reason for this process was that bacteria were able to decrease their metabolism in order to survive stressful conditions, an action which he termed as “playing dead” to survive antimicrobial agents. Wang and Wood (2011) identified the toxin-antitoxin (TA) systems as key mechanisms in regulating bacterial stress responses. TA systems are often composed of two genes in an operon encoding a stable toxin that disrupts an essential cellular process and deactivate cellular metabolism. TA systems also regulate the general stress response (GSR), GSR, a mechanism mediated by the antitoxin MqsA. This has been consequently associated with a significant reduction in bacterial growth rate. This helps bacteria to survive various environmental stresses such as long periods of starvation. Furthermore,

under specific stresses such as toxin accumulation, the TA mechanism reduces growth and instead directs bacterial metabolism towards persister cells (Wang and Wood, 2011)

This persistent form of tolerance to antibiotics is known as drug indifference. It is when a subpopulation of the bacterial cells refrains from undergoing active replication. Indeed, there have been clinical observations and studies into drug indifference when exposed to active antibiotics. All antibiotics function most effectively at killing bacterial cells when they are in their exponential growth phase. However, when bacterial cultures are able to stop replicating and remain in the stationary phase, they become insensitive to the antibiotics (Tuomanen et al., 1986; Xie et al., 2005).

While certain antibiotics such as streptomycin and ciprofloxacin have been observed to kill bacteria in the stationary phase, their rate of mortality against bacteria was much less when compared with their effectiveness in killing exponentially growing cultures. There are a number of biological mechanisms that have been proposed to explain how the bacterial populations are able to cease their replication processes and adopt the persistent phenotypes that allows them to survive (Levin and Rozen, 2006).

A possible explanation is that during colonization, bacterial populations will often include a subset of cells that has not yet begun to replicate (Metris et al., 2005), which explains how the bacteria are able to survive when the replicating cells have been killed. Also, bacterial populations have cells that stop replicating in order to repair damaged DNA which occurs when bacteria are exposed to toxins and other stresses, hence increasing the number of bacteria that have stopped replicating. This increases the number of non-replicating cells. It has also been shown that some antibiotics are able to induce the SOS system which can stop bacterial replication (Miller et al., 2004). The SOS system is a global response to DNA damage which triggers an increased expression of the genes involved in DNA repair as well as cell survival. The SOS system increases mutation and proliferation of resistance genes and has been associated with β-lactam antibiotic resistance in MRSA (Curiolo et al., 2009). The activation of the SOS response in bacteria by antibiotics may be attributed to the nature of antibiotics having a signalling role in bacteria (Aminov, 2007).

A later study has suggested that the resistance acquired from starvation stress is actually caused by active resistant mechanisms rather than passive effects of growth arrest and restriction in metabolism by Nguyen et al. (2011). This study suggested that the mechanism through which the starved bacteria are protected against antibiotics is controlled by the starvation-signalling stringent response (Godfrey et al., 2002). Their results coincided with this mechanism as bacteria showed increased sensitivity to antibiotics when the Stringent Response was inactivated (Nguyen et al., 2011). However, this study by Nguyen et al. (2011) was carried out on *Pseudomonas aeruginosa* biofilm bacteria not staphylococci. Biofilms have been shown to be produced by *S. xylosus* (Planchon et al., 2006). This may have been visible in the aggregation of bacterial cultures in the results discussed.

In biofilms which usually have reduced nutrient access (i.e. starvation) the SR which in turn activates other systems of survival such as increasing antioxidant defences.

This might suggest that starvation induced antibiotic resistance may not be entirely dependent on the mechanisms of antibiotic resistance such as antibiotic target modification, enzymatic inactivation or efflux pumps (Nguyen et al., 2011). It must be noted that while the study by Nguyen et al. (2011) regulated their nutrient starvation models, the method for this study did not regulate the process. Thus the stress factors may have included, in addition to starvation, the accumulation of bacterial toxins and the lack of restrictions in the transfer of antibiotic resistance determinants.

Thus, it can be deduced that the starvation stress (lack of nutrients) carried out in this experiment may have elicited a combination of different mechanisms of resistance in the cultures that contributed to the antibiotic insensitivity of the starved cultures during antibiotic susceptibility testing.

3.4.2 Cultivation of cultures to be used in ^{1}H-NMR analysis

The bacteria were cultured in tryptic soy broth (TSB) instead of standard nutrient broth I for the bacterial metabolite NMR analysis. This was due to the use of TSB in other metabolomic analyses of staphylococci using ^{1}H-NMR (Xu et al., 2010) and the difficulty of

obtaining the bacteria from agar medium without also introducing agar to the samples to be analysed.

The separation of bacterial culture from the growth media was done while ensuring that the cellular metabolites remained stable and intact. Immediately after the bacterial cells were collected, they had been quenched with cold methanol (-20 °C) to stop all metabolism in the cells and preserve the metabolites to be analysed. The reason for using cold methanol was due to its high cellular permeability that allows it to quickly enter the bacterial cell walls and terminate cellular activity while keeping the cell intact (Patejko et al., 2017).

The wild-type and mutant bacterial cultures were tested for susceptibility before culturing commenced for metabolite analysis in order to confirm that they were still antibiotic susceptible and antibiotic resistant. The results in Figures 3.7 and 3.8 re-cultured from the same bacterial strains prepared in earlier experiments showed that the wild-type strain was still susceptible on tryptic soy broth while the mutant strain was still resistant. These results were observed for the 16 replicates made for the extraction procedure.

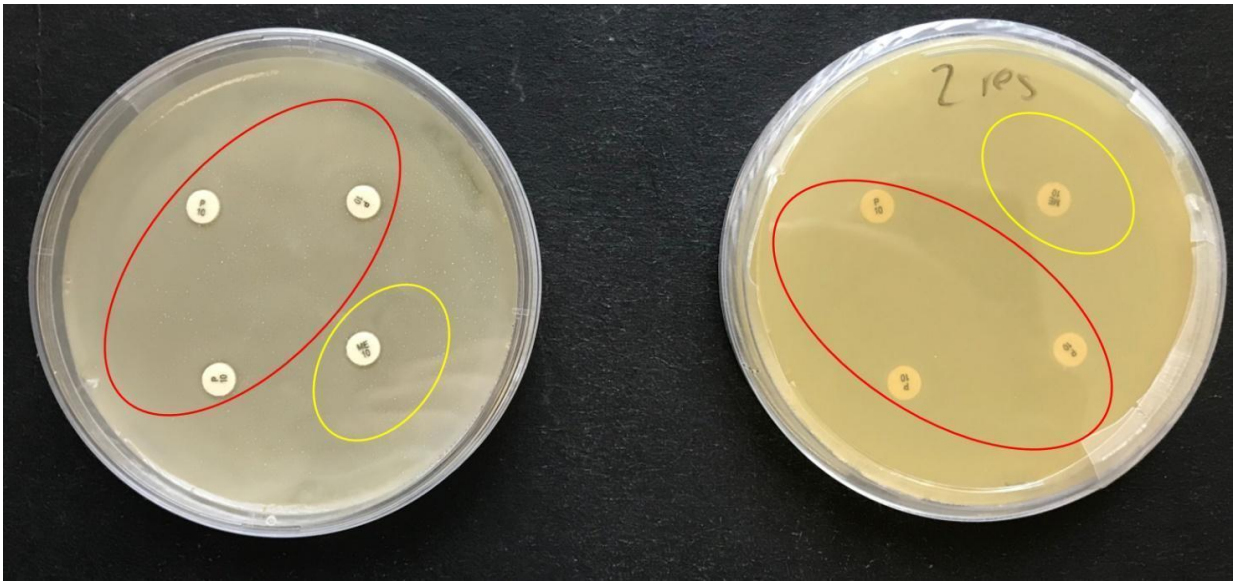


Figure 3.7 Susceptibility testing for resistant bacterial cultures in tryptic soy broth. The plate on the left is facing up while the plate on the right is face down. Red circles are penicillin discs while the yellow circles are methicillin discs

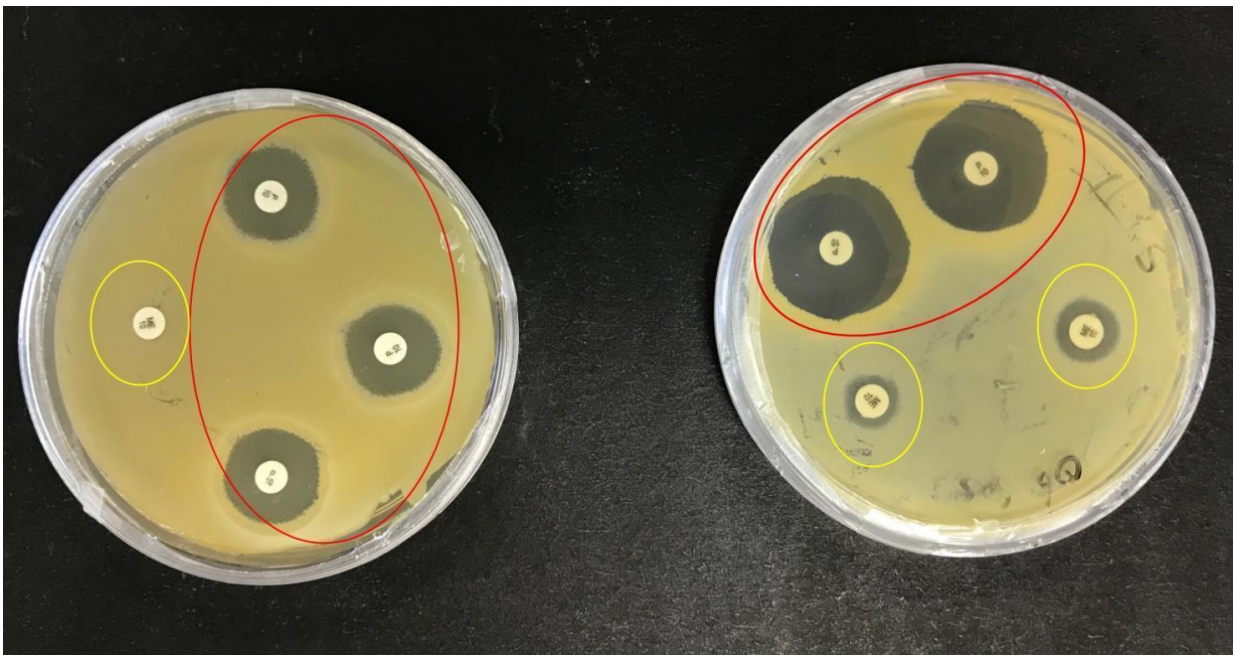


Figure 3.8 Susceptibility testing in tryptic soy broth for wild-type (susceptible) strain. The plate on the left is facing up while the plate on the right is face down. Red circles are penicillin discs while the yellow circles are methicillin disc. Considering every other antibiotic disc showed inhibition, the single methicillin disc (far left) might not have shown inhibition because it wasn't securely placed on the agar.

The only notable difference was the zone of inhibition around the methicillin antibiotic discs was much smaller compared to the zone of inhibition around the penicillin antibiotic discs (Figure 3.8). Nonetheless, there was still a certain region around every antibiotic disc around which the wild-type bacteria were unable to grow. This could have been as a result of lower methicillin solubility in the tryptic soy agar growth medium. No sources were found to explain the reason for this observation, and further susceptibility tests and follow-up analyses could not be carried out to determine the cause of this lack of inhibition. It is possible that the antibiotic disc was placed too lightly on the agar that only a small amount of antibiotic diffused into the agar.

3.4.3 ^{1}H-NMR spectral data

^{1}H-NMR spectroscopy was selected as the means of comparative metabolomic analysis particularly due to its sensitivity to analysing a wide range of metabolites simultaneously without tedious sample preparations whilst distinguishing metabolic differences `without prejudice' (Nicholson et al., 1999)

Six of the resistant mutants and 6 of the susceptible wild-type culture replicates were analysed by NMR. It was noted from all the stacked NMR spectra in Figure 3.9 that a great deal of similarities existed in the NMR signals. The extracts contained a large amount of the culture medium that was left amongst the collected bacteria cells during the separation and extraction. There were also significant similarities between the spectra from the same strain.

There were a number of differences that are consistent between all the resistant and all the susceptible spectra. Most notable is a large singlet hydrogen signal at about 3.30 ppm in all the susceptible bacterial extracts' spectra (Figure 3.9 spectra 7-11) but it is present in a much smaller concentration in all the resistant bacteria's spectra. There is also a singlet at about 3.90 ppm in all susceptible spectra but it's absent in all the resistant spectra. Another difference observed in all the resistant bacteria, downfield of the doublet at 2.35 ppm, is the presence of a singlet at about 2.40 ppm which is not present in the susceptible bacteria.

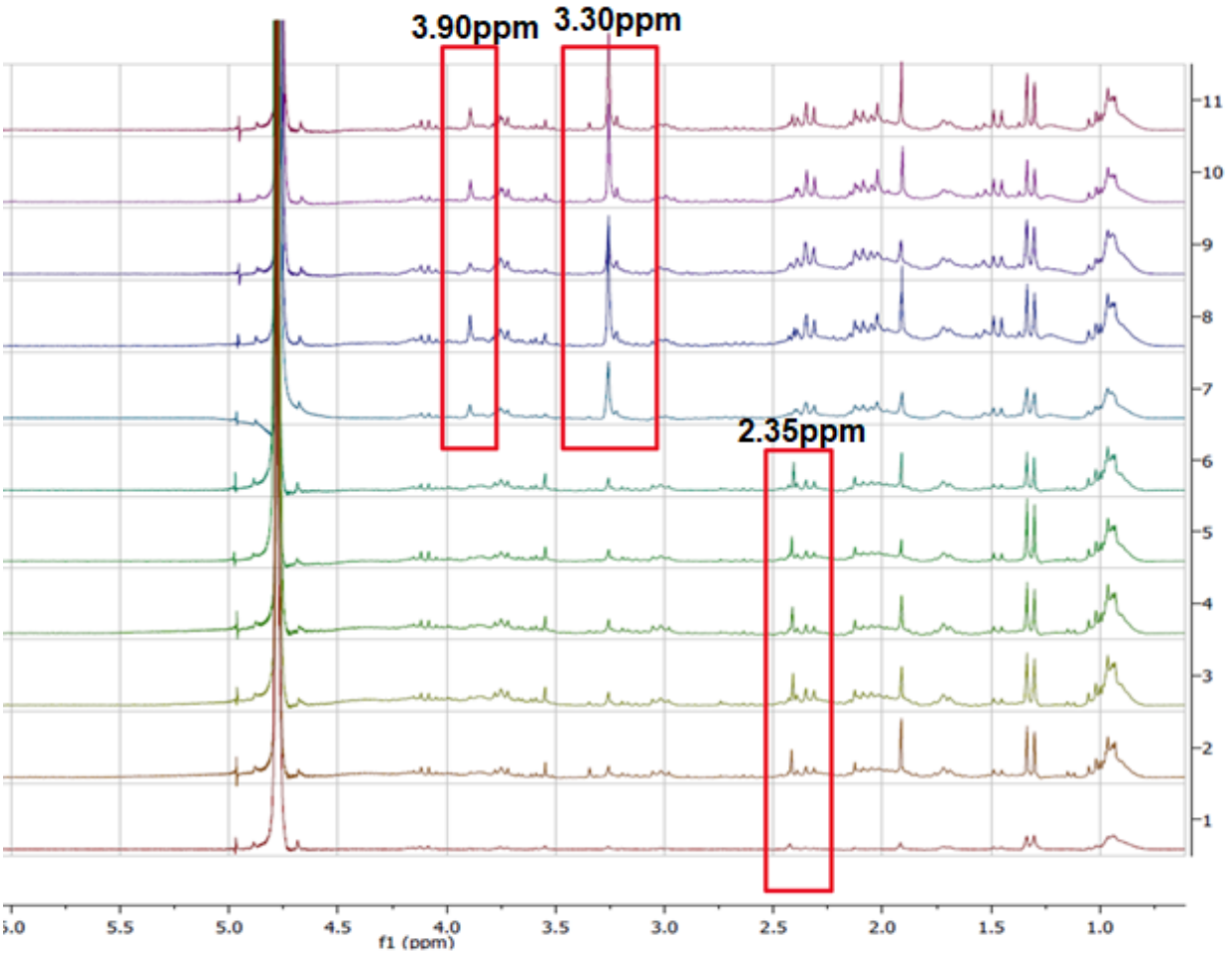


Figure 3.9 Stacked ^{1}H-NMR spectra for all 11 samples analysed. Spectra 1-6 are for the resistant cultures while spectra 7-11 are for the wild-type/susceptible cultures. The peaks shown in red boxes did not feature in the opposite strain.

It is thus clear from Figure 3.9 that there were a number of striking NMR signal differences between the two bacterial strains which suggests that the resistant strain, despite being grown from the same culture as the wild-type culture had developed a different chemical profile and different metabolites as it became resistant to antibiotics.

The control for the NMR analysis was the original TSB growth medium without any bacteria being added. The NMR spectrum of the control (blank broth) had two prominent singlets at 2.57 ppm and 3.25 ppm (Figure 3.10). These signals were not observed in any of the resistant culture's extracts, however, a prominent signal was present a little upfield from 3.25 ppm, which might indicate a small difference in the structure of the molecule. Stacking the control and susceptible extracts (Figure 3.11) did not show conclusively if the peaks at 3.25 ppm and 3.24 ppm were overlapping or two distinctive peaks. Therefore, a sample of the control was combined with a

sample of the susceptible extract (replicate no.5) and analysed together by ^{1}H-NMR to produce a combined spectrum from which it would be possible to see if the two peaks are of the same signal.

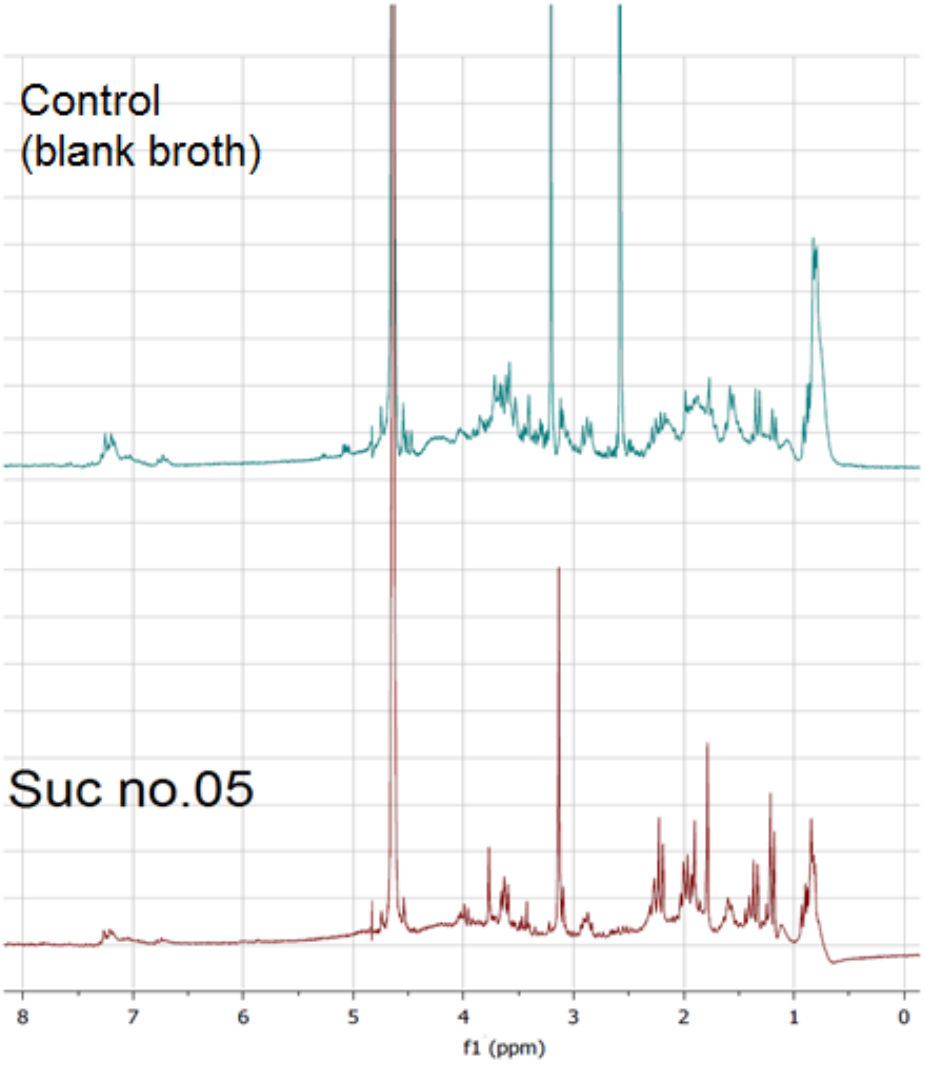


Figure 3.10 Stacked spectra for blank tryptic soy broth and susceptible bacteria extract no. 5

Figure 3.11 shows that the large singlets at 3.25 ppm in the control and 3.24 ppm in susceptible spectra were in fact two separate signals (red arrow), thus they represent two distinct compounds. This meant that the two prominent peaks on the control spectrum were most likely from a large amount of a particular compound that are taken up or modified by both resistant and susceptible bacteria cultures.

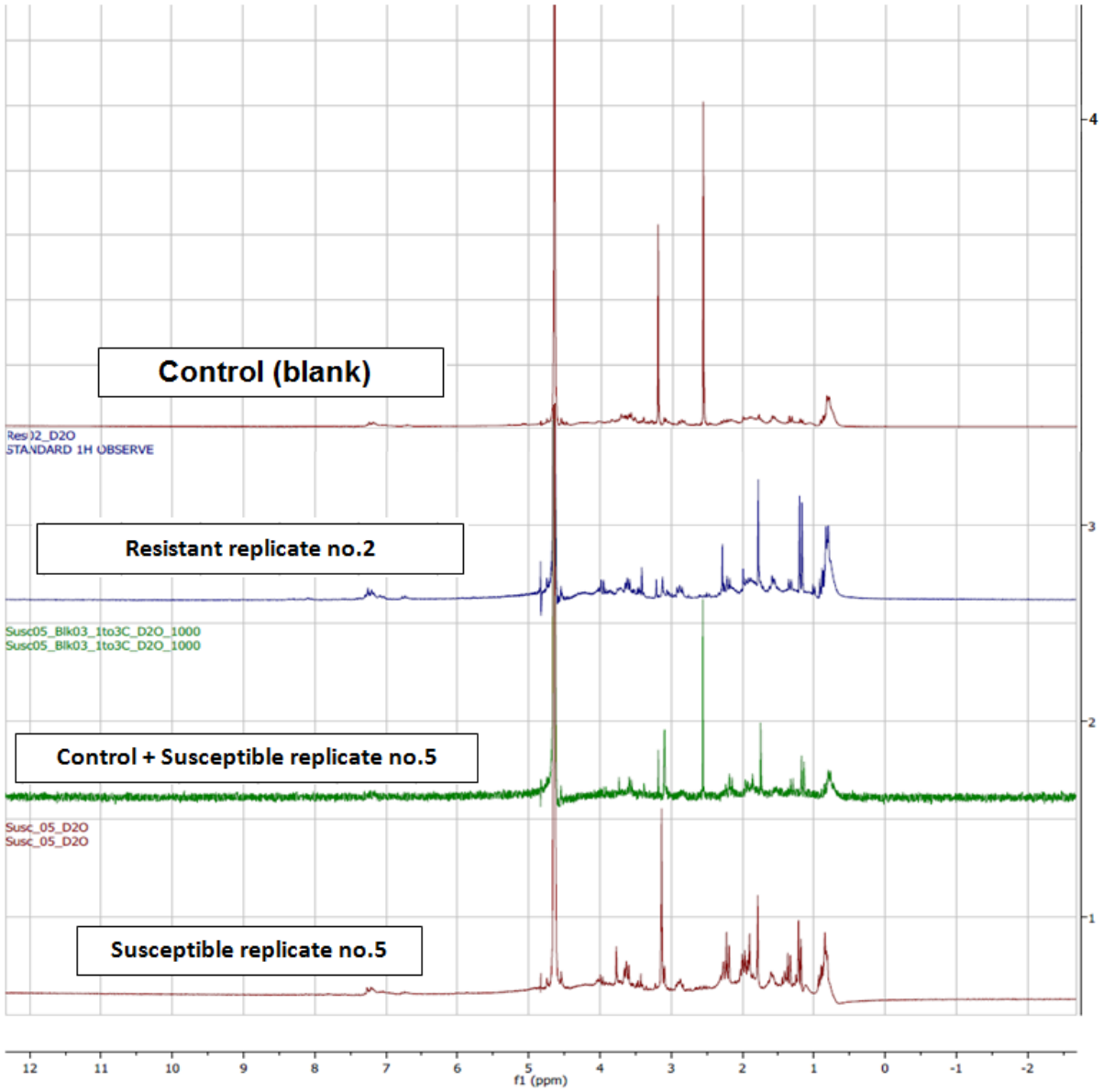


Figure 3.11 Stacked ^{1}H-NMR spectra for 1. Susceptible replicate no.5, 2. Susceptible replicate no.5 combined with control tryptic soy broth, 3. Resistant replicate no.2 and 4. Control tryptic soy broth

Since there could be more differences in the spectra of the extracts, probably not detected by visual analysis of the data, further metabolomic analyses of the two bacterial strains' extracts were done by multivariate analysis methods.

3.4.4 Statistical multivariate analyses

The first multivariate analysis done on the spectral data was a principal component analysis (PCA). This was used to determine if the chemical differences between the two strains were

significantly discernible such that consistent statistical differences could be identified (Figure 3.12). The PCA analysis was done by using the data processing program for metabolomic analyses, SIMCA. A PCA data analysis is completely unsupervised and therefore done without indicating which extracts were from the susceptible or resistant bacteria (Heyman and Meyer, 2012).

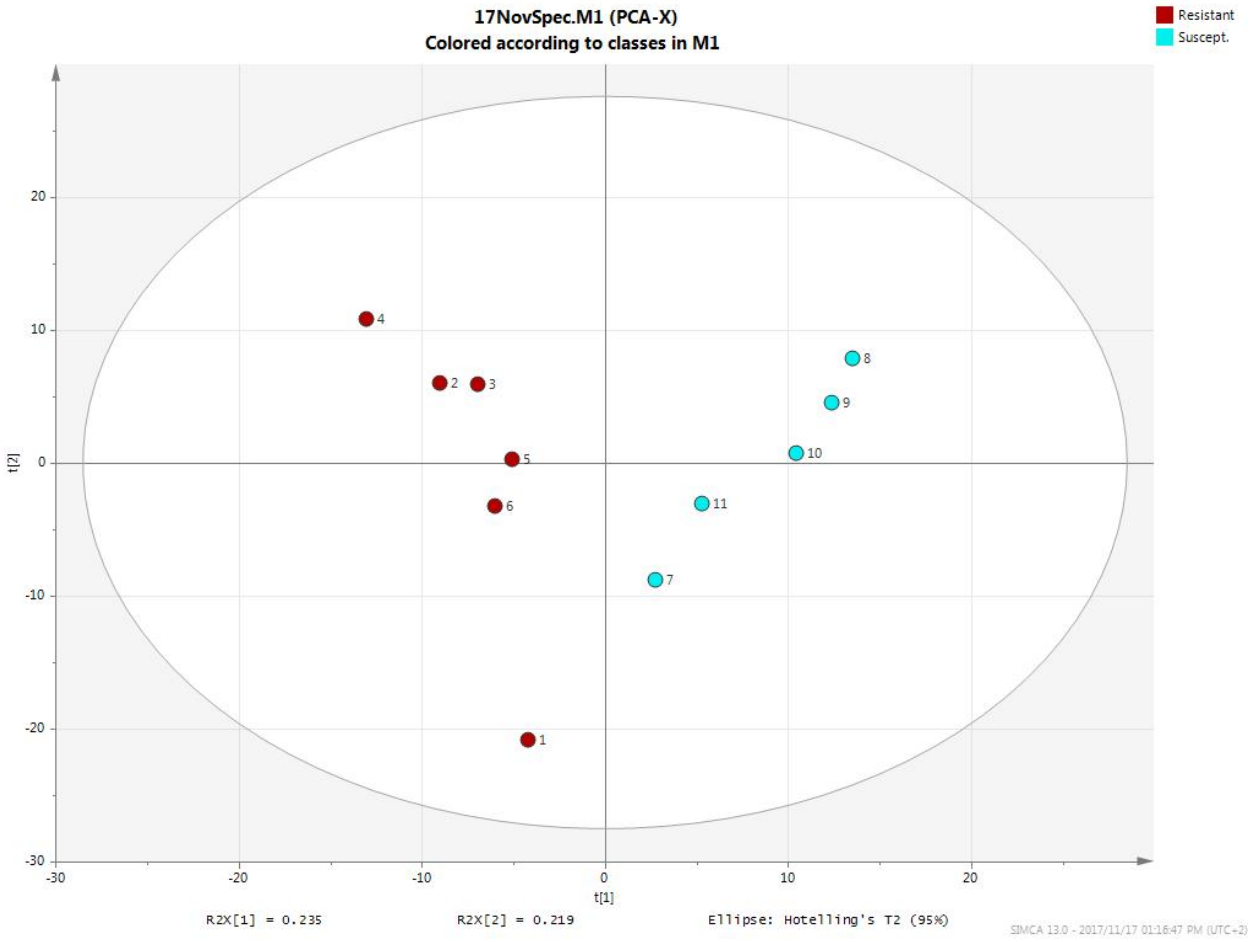


Figure 3.12 PCA plot for 6 resistant/mutant bacteria spectra (red) and 5 susceptible/wild-type bacteria spectra (blue) shows an unsupervised discernment between the two strains. There were only 5 samples analysed for the susceptible extract. Susceptible replicate no.3 was not included in the PCA and OPLS analyses as the vial was found to have cracked during storage which might have contaminated the sample.

The results of the PCA analysis clearly distinguished between the resistant and susceptible/wild-type bacteria by grouping them into two discrete chemotypes. Thus the wild-type antibiotic susceptible *S. xylosus* could be differentiated from its antibiotic-resistant strain that had been grown from the same reference culture.

An OPLS analysis was also carried out using SIMCA in which information on the identities of the resistant and susceptible extract data was specified. The OPLS analysis (Figure 3.13) also recognised that the resistant and susceptible chemical profiles were different. Resistant replicates 3, 5, and 6 showed a significant overlap on the OPLS plot which meant that they contained similar compounds with similar concentrations. The same was noted for susceptible replicates 2 and 4. The other replicates showed differences in concentrations. This could possibly be due to natural biological variance and/or to the inevitable human errors in the execution of the experimental procedures.

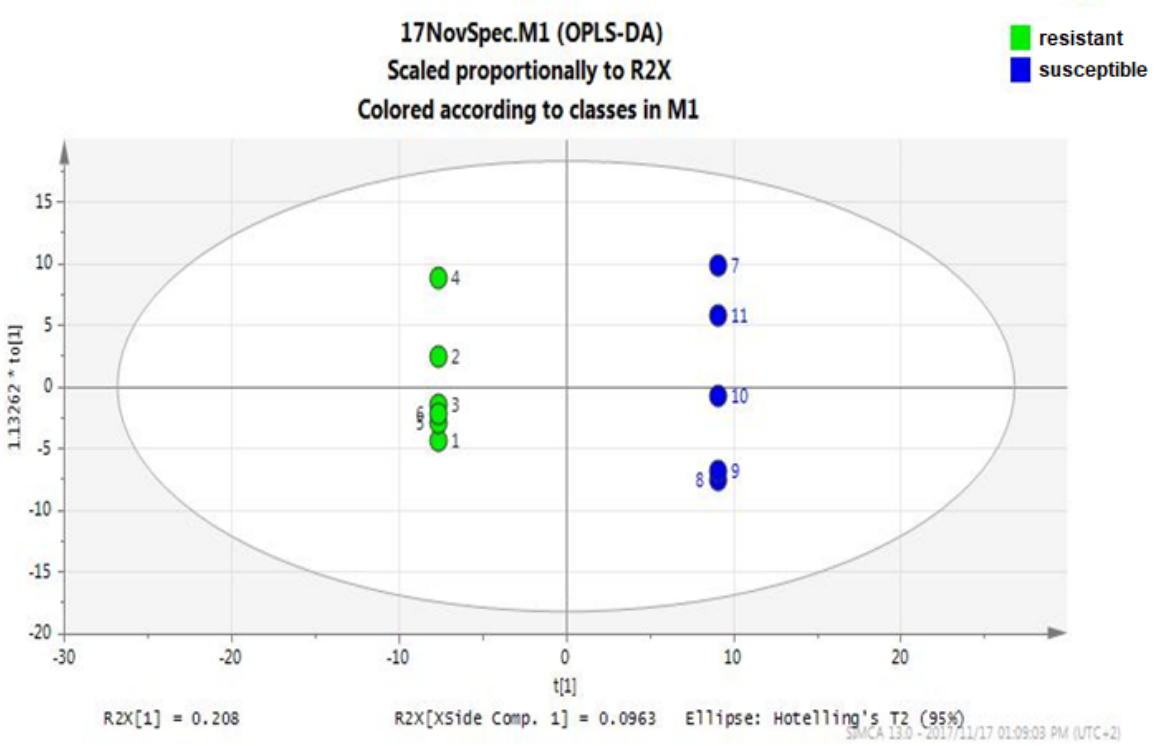


Figure 3.13 OPLS analysis of the resistant and susceptible NMR data.

A contribution plot (Figure 3.14) provides an indication of the type of compounds that contributes mostly to the groupings (separation) of the samples. This is based on the abundance of specific ^{1}H chemical signals that are represented as peaks in the ^{1}H-NMR data. The contribution plot of the susceptible and resistant bacterial extracts showed that a number of compounds between 1.0 ppm and 3.0 ppm were present in the susceptible bacterial cultures at a much higher concentration than in the resistant bacterial cultures. In turn, the resistant bacteria showed to have a much higher proportion of metabolites that

were detected between 4.0 ppm and 5.7 ppm. The resistant bacteria also seemed to show a higher proportion of compounds at 8.0 ppm, 8.5 ppm and 9.0 ppm.

In Figure 3.14, the bin region between 3.0 and 3.3 ppm for the susceptible extract shows the strongest Hydrogen signal in the contribution plot. This region also corresponds to the significant peak in figure 3.9 that featured prominently in all the susceptible peaks. The second strong Hydrogen signal at 3.9 ppm in Figure 3.14 The low signal for the hydrogen peak at 3.3 may be attached

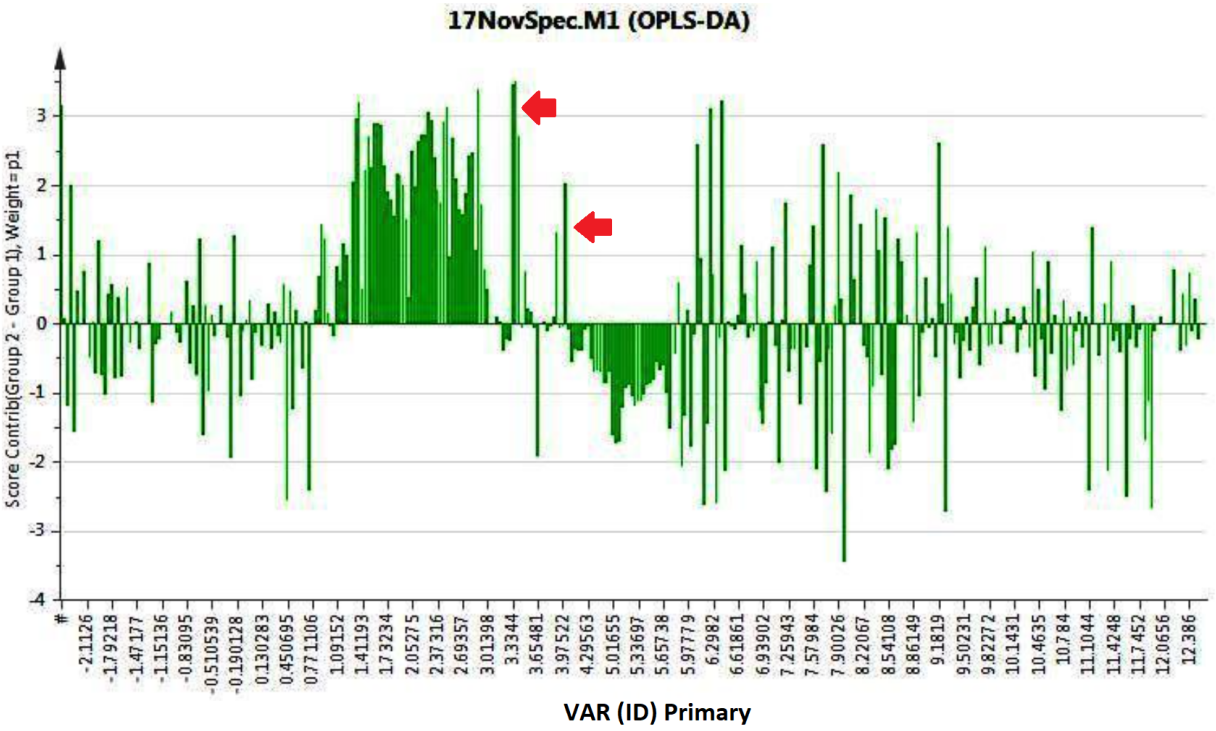


Figure 3.14 The contribution plot with the combined data from the 5 susceptible spectra (top) and the combined data from the 6 resistant spectra (bottom). The distinctive hydrogen peaks seen in the susceptible stain in stacked spectra (Figure 3.9) are shown by red arrows at 3.9 ppm and 3.3 ppm

The distribution of metabolites in the contribution plot shows a relatively equal distribution with only a few of the samples being similar in chemistry and concentration. Bacterial metabolites tend to serve multiple purposes including simultaneously facilitating a number of bacterial biochemical reactions (Dordet-Frisoni et al., 2007).

The region between 4.0 ppm and 5.7 ppm shows a group of compounds significantly more abundant in the resistant strain than the susceptible strain. This may be due to an increase

in the production of molecules and signalling factors that protect or conceal certain targets. Indeed there could be a long list of possible mechanisms that confer antibiotic resistance to the resistant strain. The full analysis of the exact metabolite compounds may require more sensitive NMR analysis (600 MHz and above) as suggested by Nicholson et al. (1999).

Chapter 4: Isolation and Identification of Staphyloxanthin and its precursors

4.1 Introduction

Following the successful completion of the first two objectives of this study, which were creating an antibiotic resistant strain from an antibiotic susceptible WT and then comparing their metabolomic profiles, the third and final aim of this study was the isolation of a potential biomarker for antibiotic resistance. According to the International Programme on Chemical Safety, led by the World Health Organization (WHO), a biomarker is "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (WHO, 2001). They stated that biomarkers can further be classified as either a marker of exposure, effect and susceptibility.

The persistent problem of antibiotic resistant bacteria is that they cannot be easily diagnosed, thus during the treatment of antibiotic resistant infections, precious time can be lost (Spellberg and Gilbert, 2014). Resistant bacteria are often similar in appearance to susceptible strains so the identification of a unique biomarker could be an invaluable tool in the fight against antibiotic resistance

A potential biomarker was tentatively identified when the original WT strain of *S. xylosus* and the consequently resistant strain both were left on the same open counter a few days. The WT strain which was still susceptible to antibiotics turned to a bright yellow-orange colour while the resistant strain did not. It was postulated that this orange pigment was a carotenoid pigment like staphyloxanthin or a precursor of it, as was previously shown to be produced by various staphylococci including *S. xylosus* (Seel et al. 2020).

S. xylosus produces various pigments at lower temperatures, some of which are characteristically found in the various food products made through *S. xylosus* (Schleifer and Koos, 1975). These pigments are often produced at lower temperatures of about 10^{o}C which explains the pigment production by the susceptible strain in Figure 4.1. The important question is why did only the susceptible strain produce it, but this could be a result of a myriad of effects which will be discussed in this chapter.

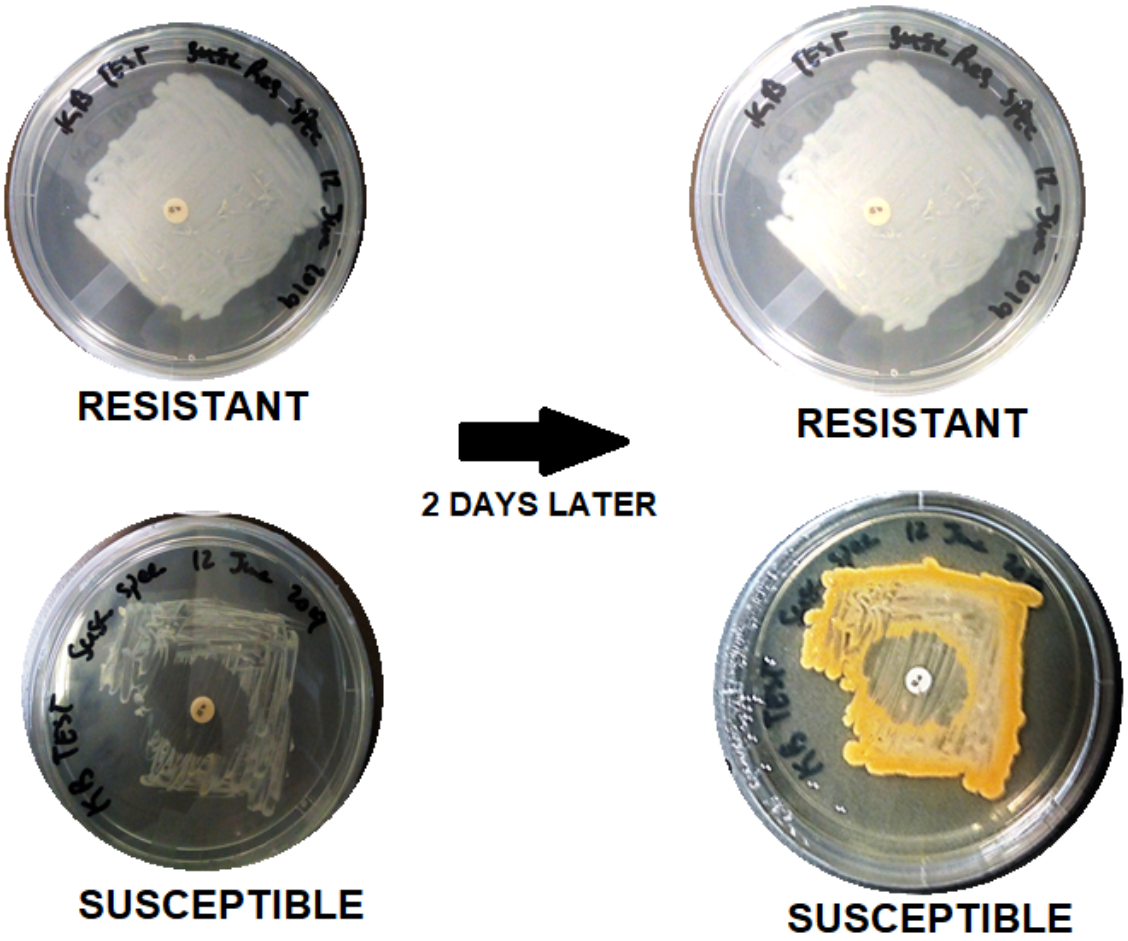


Figure 4.1: KB susceptibility test which was left unattended on a lab bench for 2 days. The susceptible culture turned to a bright orange while the resistant culture remained white

4.2 Bacterial carotenoids

Staphylococcus aureus was named by Rosenbach (1884) after isolating the pigmented pathogen from human skin. It received the species name 'aureus' which means gold because of the golden colour of the colonies. Staphyloxanthin was a key element in the identification of *S. aureus*. Staphyloxanthin is produced through a long biosynthetic process in which two C5 isopentyl pyrophosphate (IPP) molecules are combined to form farnesyl diphosphate. The next synthesised pigment is 4,4'-diaponeurosporene, with a bright yellow colour (Figure 4.2). After consequent modification with the addition of a carboxylic acid functional group (-COOH) to make 4,4'-diaponeurosporenoic acid, a glucosyl group is esterified with the triterpenoid

carotenoid carboxylic acid group before it reacts with a C_{15} fatty acid to form a second ester link to yield the final product (Marshall and Wilmoth, 1985).

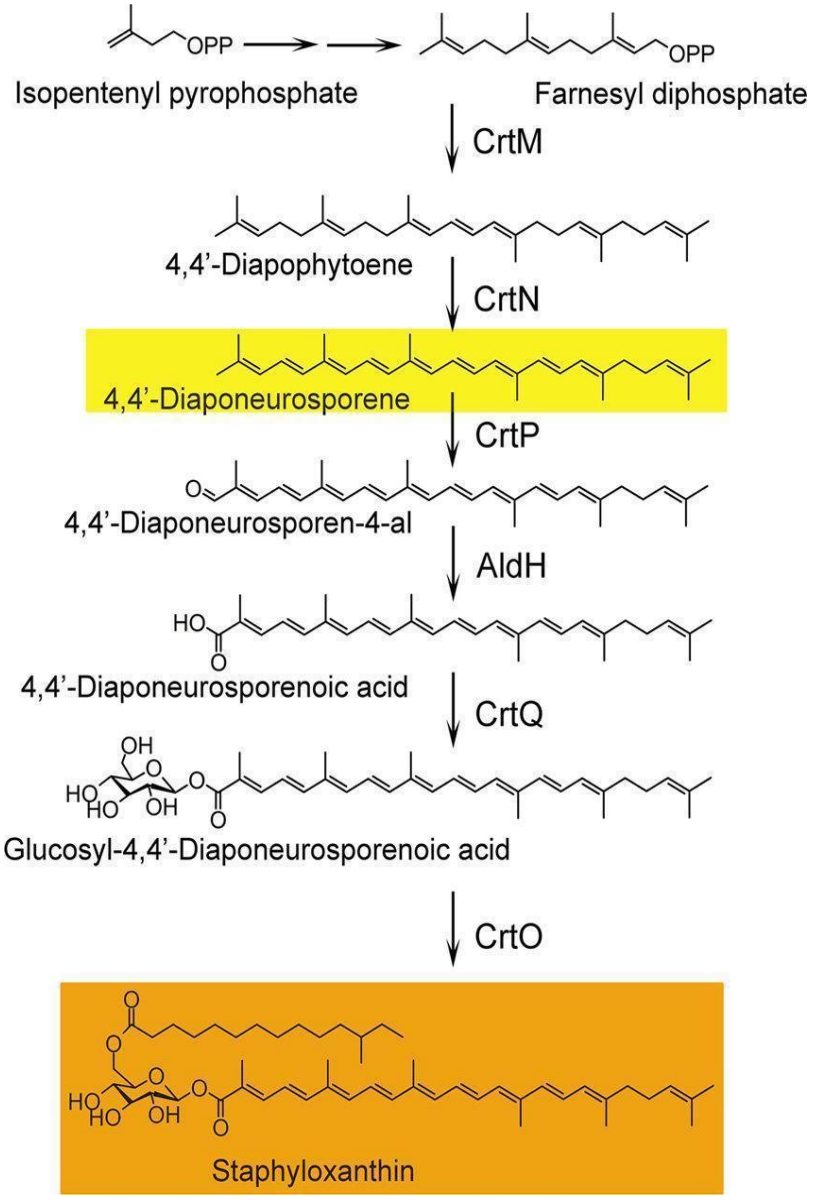


Figure 4.2: Biosynthesis of staphyloxanthin in staphylococci (Gao et al., 2017). The coloured boxes indicate the colour of the compounds.

Staphyloxanthin production in *S. aureus* is controlled by the sigma factor B (σB) which in-turn regulates the crtMN operon (Wieland et al., 1994). CspA (cold shock protein A) which is activated during cold stress regulates the carotenoid-linked sigma factor σB which could explain the *S. xylosus* cultures turning yellow/orange when left at low temperatures (Figure

4.1) (Donegan et al., 2019). Staphyloxanthin plays a key role in allowing bacteria to withstand ultraviolet radiation as well as damage from radical oxidative species. Bacteria that produce staphyloxanthin were found to survive oxidative stress better (Clauditz et al., 2006).

Katzif et al. (2005) found that mutations of the cspA gene were also linked to a lower rate of crtN expression, a gene associated with the production of staphyloxanthin in *S. aureus*. *S. xylosus* also produces staphyloxanthin and various staphyloxanthin-like derivatives which have been linked to increased membrane fluidity and increased survivability (Seel et al., 2021). In their study of carotenoids regulating membrane fluidity in *S. xylosus*, Seer et al. (2021) noted an increase in carotenoid production during cold stress as a result of increased expression of the cspA gene.

This extended endurance contributes to the development of antibiotic resistance as staphyloxanthin-producing bacteria would be able to acquire or share mobile genetic elements with antibiotic resistance genes. In hospital environments where there is a high proliferation of antibiotic resistance determinants, staphyloxanthin could help make previously susceptible bacteria survive for longer thereby allowing the bacteria to adapt to or acquire antibiotic resistance genes (Xia et al., 2016).

Resistance and persistence have complex mechanisms. Having been previously exposed to starvation stress, the resistant strain could have reached its stationary phase faster in order to preserve energy (Tuomanen et al., 1986). That would have resulted in a lower production of staphyloxanthin. The lag phase would mean that bacteria were more dormant and thus less likely to produce compounds like staphyloxanthin that require more energy. Figure 4.3 shows the susceptible and resistant cultures that were placed in a refrigerator at 3-5^{o}C and slowly grew under these conditions. The susceptible strain turned orange while the resistant one turned yellow. The orange in the susceptible strain could have been due to staphyloxanthin while the yellow in the resistant could have been 4,4’-diaponeurosporene based on the Gao et al. (2017)'s staphyloxanthin biosynthetic pathway (Figure 4.2). This result was also observed in the *S. xylosus* bacteria of three other susceptible cultures and three resistant cultures.

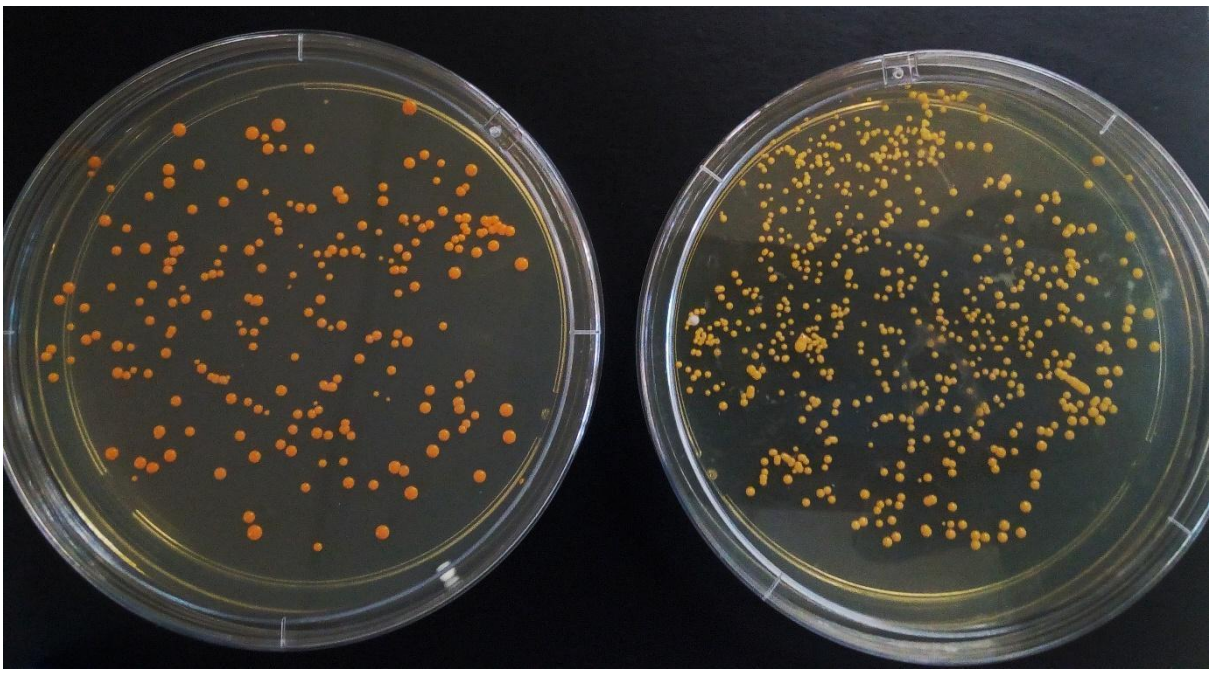


Figure 4.3: Susceptible *S. xylosus* strain (left) and the resistant *S. xylosus* strain (right) after four months of incubation inside a dark fridge at 3-5ºC.

As the resistant strain slowed down its metabolism, it may have deactivated the CrtP factor in the biosynthetic pathway which is synthesised after the bright yellow 4,4'-diaponeurosporene (Figure 4.2).

Staphyloxanthin is a non-essential metabolite involved in the staphylococcal virulence mechanism by protecting infecting bacterial pathogens from phagocytosis after becoming engulfed by macrophages (Olivier et al., 2009). Staphyloxanthin also assists in staphylococcus virulence by protecting bacteria against hypochlorous acid, a radical oxidative species produced by neutrophils which are an important part of the innate immune system (Lui et al., 2005).

As discussed in Chapters 2 and 3, bacterial resistance to antibiotics comes as a result of stresses on bacteria. Antibiotics which restrict and disrupt essential processes in bacterial cells also cause stress which in turn causes the bacteria to mutate, adapt and develop resistance quicker. However, while staphyloxanthin is a key metabolite in helping bacteria survive oxidative stress, the pigment is not essential for bacterial survival. By targeting and limiting staphyloxanthin production in bacteria, the bacteria will be losing a key component in its defence but not experience the same level of stress as staphyloxanthin is not essential in any primary metabolic processes.

Thus staphyloxanthin is an important target to fighting antibiotic resistance. Targeting the production of this pigment would not only weaken the bacteria's endurance and survival, it would also limit its pathogenesis. Plant chemotherapeutics have been found to be effective in inhibiting staphyloxanthin growth. Lee et al. (2012) found that plant flavones such as luteolin can reduce the production of staphyloxanthin without inhibiting *S. aureus* growth.

The comparative (susceptible vs. resistant) model developed in this study could allow for the screening of different plant extracts to compare the synthesis of staphyloxanthin.

The third objective of this study was to isolate and identify potential biomarkers and unique targets. Having provisionally identified staphyloxanthin as a potential biomarker, the next objective was to isolate and confirm its presence and/or its biosynthetic precursors in the susceptible strain.

4.3 Methods

For the yellow/orange pigment to serve as a potential biomarker, it had to be uniquely produced by the antibiotic susceptible strain of the bacteria, but not by the antibiotic resistant strain. To confirm this, both bacterial strains were re-cultured in both liquid broth and agar as described in Chapter 3 and exposed to the stress that would stimulate the production of staphyloxanthin. To replicate and confirm that only the susceptible strain produced the yellow/orange pigment while the resistant couldn't (Figure 4.1), the susceptible and resistant strains were re-cultured in duplicates on tryptic soy agar plates and incubated for 24 hours at 36^{o}C before being left under a warm light for 48 hours.

The production of the yellow/orange pigment was not significant under these culturing conditions on the plates. It was later noted that cultures that had been stored in much lower temperatures for longer periods of time were observed to produce more yellow/orange pigment. Two flasks containing a susceptible strain and two containing the resistant which had previously been prepared for re-culturing experiments were then kept for 3 months in a refrigerator at 3-5^{o}C.

Afterwards, the bacteria were centrifuged at 10 000 rpm to separate the broth from the bacteria. The bacterial pellet was then washed in methanol to remove the pigment from the

cultures. These methanolic extracts were then moved to foil-covered test tubes in order to protect the light-sensitive carotenoids.

The methanolic extracts from both resistant and susceptible cultures were then analysed by proton NMR at 200 MHz (Varian) in deuterated chloroform ($CDCl_3$) and by liquid chromatography mass spectrometry LC-MS). The LC-MS instrument was a 1260 Infinity HPLC coupled to Bruker AmaZon SL ion trap MS (Agilent Technologies, USA). The mobile phase used for the HPLC analysis was an acetonitrile-water eluent system. The method developed for the LC-MS analysis involved a reverse-phase C18 column which was used for the HPLC separation with the first 10 min being 98% acetonitrile, changed to 90% acetonitrile from 10 to 20 min. then the analysis was continued to 100% methanol from 20 min until 38 min before going back to 98% acetonitrile for the last 2 min. The mass spectrum analysis was carried out at a nebulizer pressure of 7.3 psi with a dry gas flow rate of 4 l/min and a dry temperature of 180^{o}C The mass spectrum analysis was from a range of 100 to 1200 m/z Both extract analyses were carried out at a sample concentration of 1 mg/ml.

In order to enhance the concentration of metabolites, the amount of bacteria cultured was increased 40-fold. In order to separate the yellow/orange pigment from the different metabolites, semi-preparative thin layer chromatography (TLC , Silica gel 60 F254 , Merck Germany) was used. The first TLC was developed with a solvent solution of n-butanol:ethanol:water (4:1:1) (Gupta et al., 2019). The silica was then scraped off in bands and dissolved in methanol. These extracts were then concentrated using a GeneVac (EZ-2 series, SP Scientific, England) to remove most of the methanol, re-dissolved in fresh methanol at 1 mg/ml and analysed by gas chromatography-mass spectrometry (GC-MS-QP2010, Shimadzu, Japan) The GC-MS used an AOC-20i autosampler with the samples being injected by a 10 µL syringe which was washed before every sample injection with GC-MS grade hexane, dichloromethane and methanol. The GC column was a Rtx-5MS column with a length of 29.3 m, a diameter of 0.25 µm and a thickness of 0.25 µm. The column oven was set to 60°C at the beginning of the analysis which was held for 2 min. The column temperature was then ramped up at a rate 10°C per min to 270°C - which was held for 5 min. The second temperature ramp was from 25°C per min to 300°C and held for 5 min. The injection temperature for the GC was 250°C with the total column run time being 34.20 min.

The mass spectrum (MS) for the GC-MS had an ion source temperature of 250°C and an interface temperature of 270°C. The MS detector voltage was set to 0.1 kV with a start time of 3.50 min and an end time of 34.20 min. The scan speed was 2000 at a mass hertz (m/z) ratio between 50.00 aum and 600 aum. The MS spectra were compared to reference spectra on the National Institute of Standards and Technology (NIST 14) database (NIST, USA).

4.4 Results and Discussion

4.4.1 TLC purification of bacterial cultures and ^{1}H-NMR analyses

The result of the cultures stored at 36^{o}C and then at 28^{o}C are shown in Figure 4.4. A light yellow colour can be seen in the susceptible strain while the resistant strain remained white.

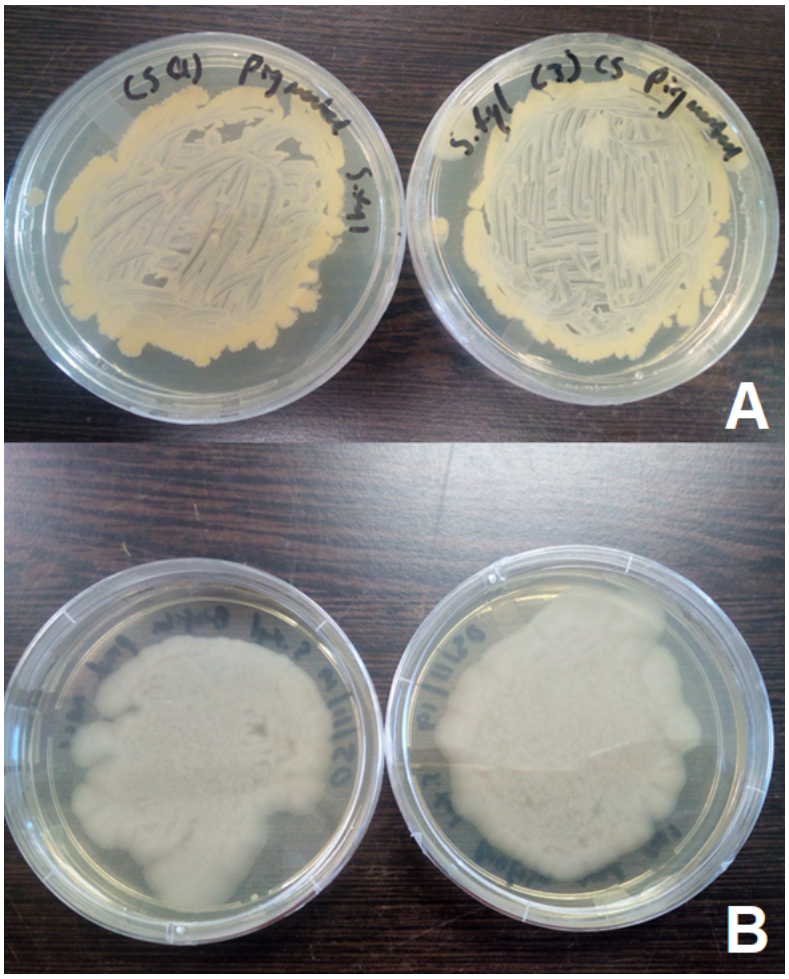


Figure 4.4: Re-culturing the susceptible (A) and resistant (B) strains. All four plates were incubated at 36^{o}C for 48 hours before being left in a phytotron at 28^{o}C under 490 nm light for another 48 hours. The susceptible strains produced a light yellow pigment which was not produced by the resistant strains.

The flasks containing the susceptible concentrated bacterial broth obtained from culturing *S. xylosus* in tryptic soy broth, is shown in Figure 4.5. The colour differences between the susceptible culture (yellow-orange) and the resistant culture which didn't show as much pigment production.

Figure 4.5: Tryptic soy broth inoculated with the resistant strain shown on the left and with the susceptible *S. xylosus* strain on the right, after 3 months of refrigeration at 3-5^{o}C and concentration at reduced pressure in a GeneVac (EZ-2 series, SP Scientific, England).

In the qualitative TLC separations done on the pigment extract, some of the bands appeared to be very faint suggesting that the corresponding metabolites were not being produced in high quantities. To elucidate these metabolites, the quantity of bacterial culture was upscaled in order to produce more metabolites and obtain higher concentrations for analysis. This was done by re-culturing the susceptible sample 5 in 40 conical flasks containing 300 ml of tryptic soy broth each, some of which are shown in Figure 4.6.

Figure 4.6: A) Some of the 40, 500 ml volumetric flasks with 250 ml of tryptic soy broth immediately after inoculation with the susceptible *S. xylosus* strain. B) Cultures after 48 hours of incubation at 38ºC.

The bacterial cultures were then transferred to GeneVac flasks and centrifuged for 30 min to separate the bacterial pellets from the broth (Figure 4.7). The pigment collected in methanol was kept in a foil-covered container to limit exposure to light as the yellow/orange pigment could be staphyloxanthin or a precursor of it which are known to be sensitive to light (Barretto and Vootla, 2018).

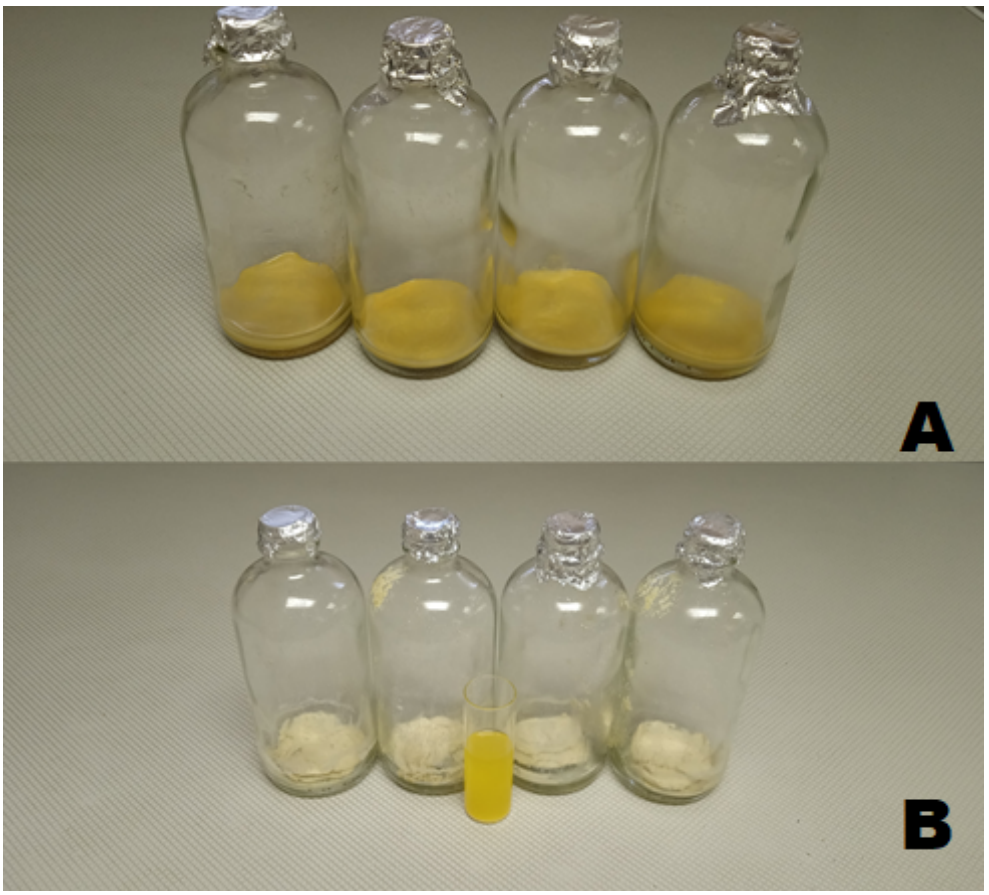


Figure 4.7: A) Bright yellow bacterial pellet of separated from the 40 conical flasks by centrifugation in the GeneVac. B) Collection of pigment in a glass vial after washing bacterial pellet with analytical grade methanol and the now white remaining bacterial pellet in the flasks.

After the upscaling of bacteria, the methanolic extracts were separated on TLC sheets (Merck) with an eluent system of n-butanol:ethanol:water (4:1:1) (Gupta et al., 2019) as shown in Figure 4.8. The TLC showed good separation of compound-bands. However the concentrations of the individual bands were too low to analyse in the 200 MHz NMR spectrometer and the extract concentrations applied to the plate were increased twenty-fold (Figure 4.8).

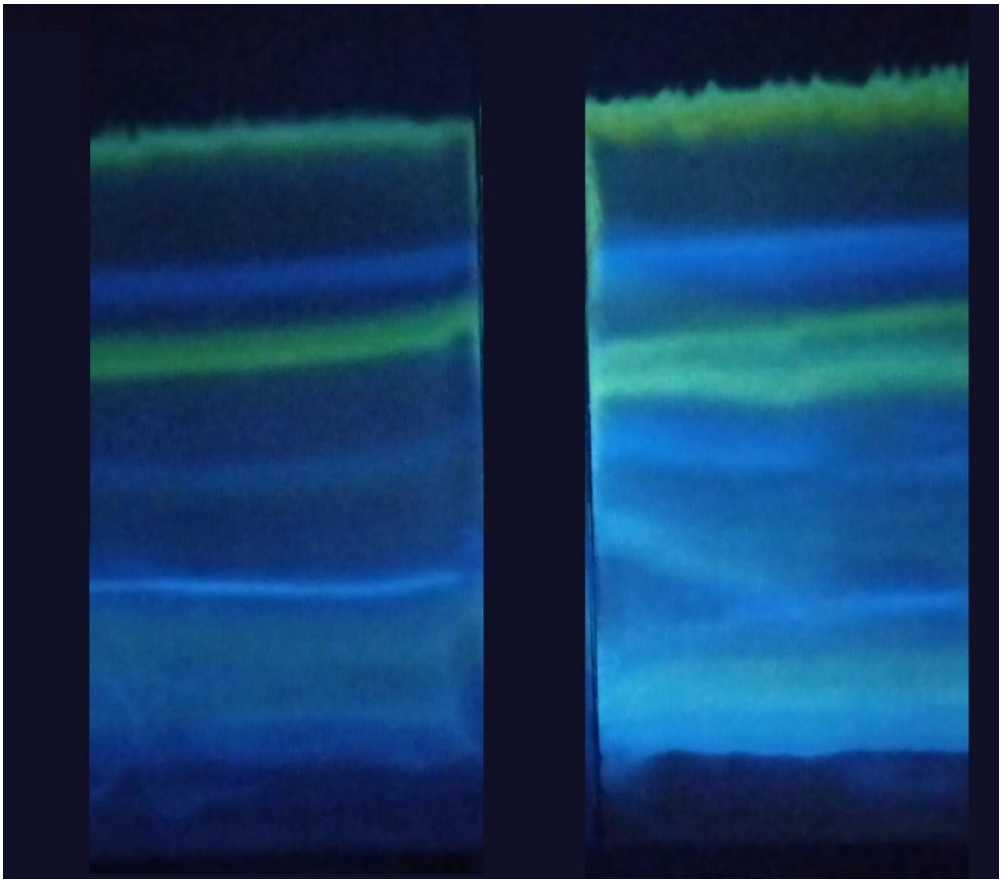


Figure 4.8: Methanol extracts from the bulk analysis culture eluted by TLC and shown under long UV wavelength (366 nm). The top bands were scraped off and analysed by NMR, GC-MS and LC-MS.

After the extract was developed on a TLC plate, the 3 main compound-bands were scraped off and the silica scraping transferred to 3 test tubes. These test tubes were left to dry overnight in a dark cabinet and analysed by ^{1}H-NMR spectrometry in deuterated chloroform (Figure 4.9).

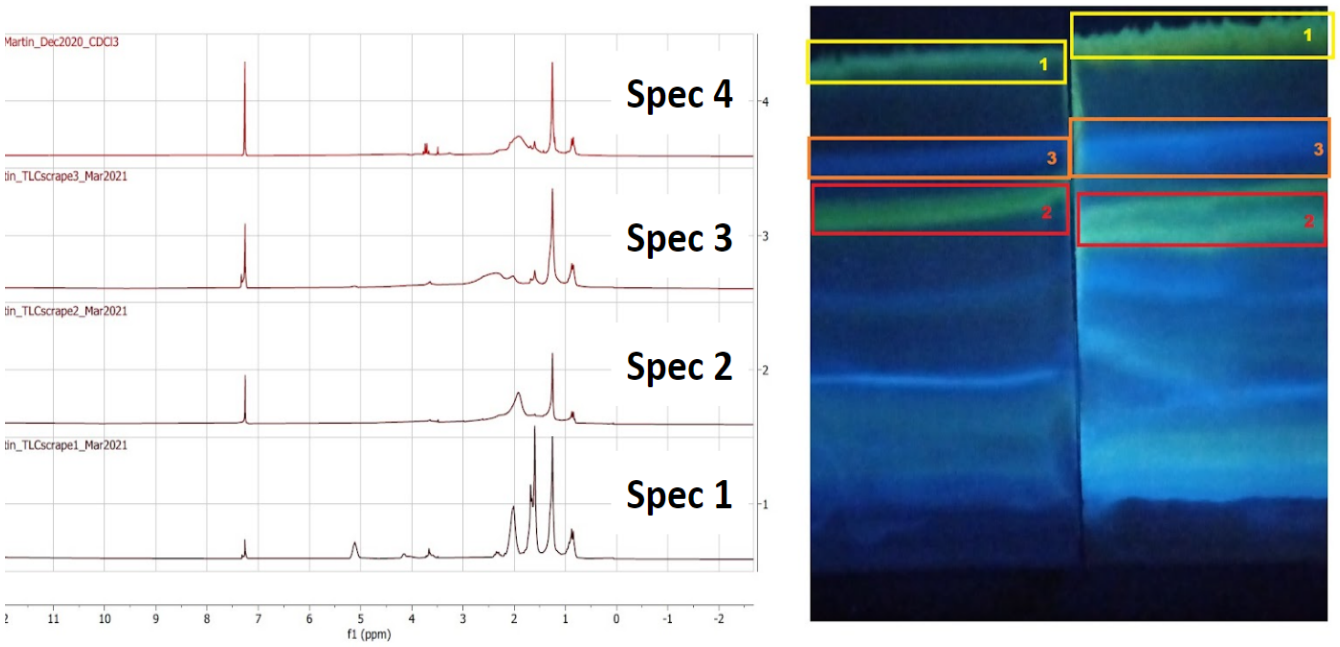


Figure 4.9: Upscaled TLCs shown on the right under long UV wavelength (366 nm) with regions scraped off the TLC plates eluted with 4:1:1, n-butanol:ethanol:water (Gupta et al., 2019). The ^{1}H-NMR spectra (left) named Spec1, Spec2 and Spec3 were collected from the bands marked 1, 2, and 3 respectively on the TLC plates on the right. Spec4 represents the NMR spectrum of the full methanolic extract of the susceptible strain.

A number of peaks observed in the bacterial extract of the susceptible strain (Figure 4.9, Spec4), were also observed in Spec1 which came from the extract from the same strain, but separated by TLC. The prominent singlet peak at 1.3 ppm observed in the analysis of the original susceptible strain (Spec4) was also observed in all the spectra of the TLC separated bacterial extracts (Spec1, Spec2 and Spec 3)

The samples purified by TLC were then analysed by liquid chromatography – mass spectrometry (LC-MS) and finally gas chromatography – mass spectrometry (GC-MS).

4.4.2 ^{1}H-NMR analysis

The ^{1}H-NMR spectrum from Spec1 (Figure 4.9) was further analysed with peaks of significance being annotated and integrated in Figure 4.10. The area under each peak was also determined by integration.

^{1}H NMR (200 MHz, $CDCl_3$) δ 5.42 (s, 1H), 5.11 (d, J = 8.0 Hz, 14H), 4.23 – 3.99 (m, 9H), 3.76 – 3.50 (m, 2H), 3.37 (d, J = 6.8 Hz, 0H), 2.32 (q, J = 7.9 Hz, 4H), 2.12 – 1.93 (m, 59H), 1.63 (d, J = 16.1 Hz, 94H), 1.25 (s, 34H), 0.89 (s, 10H), 0.89 – 0.77 (m, 7H).

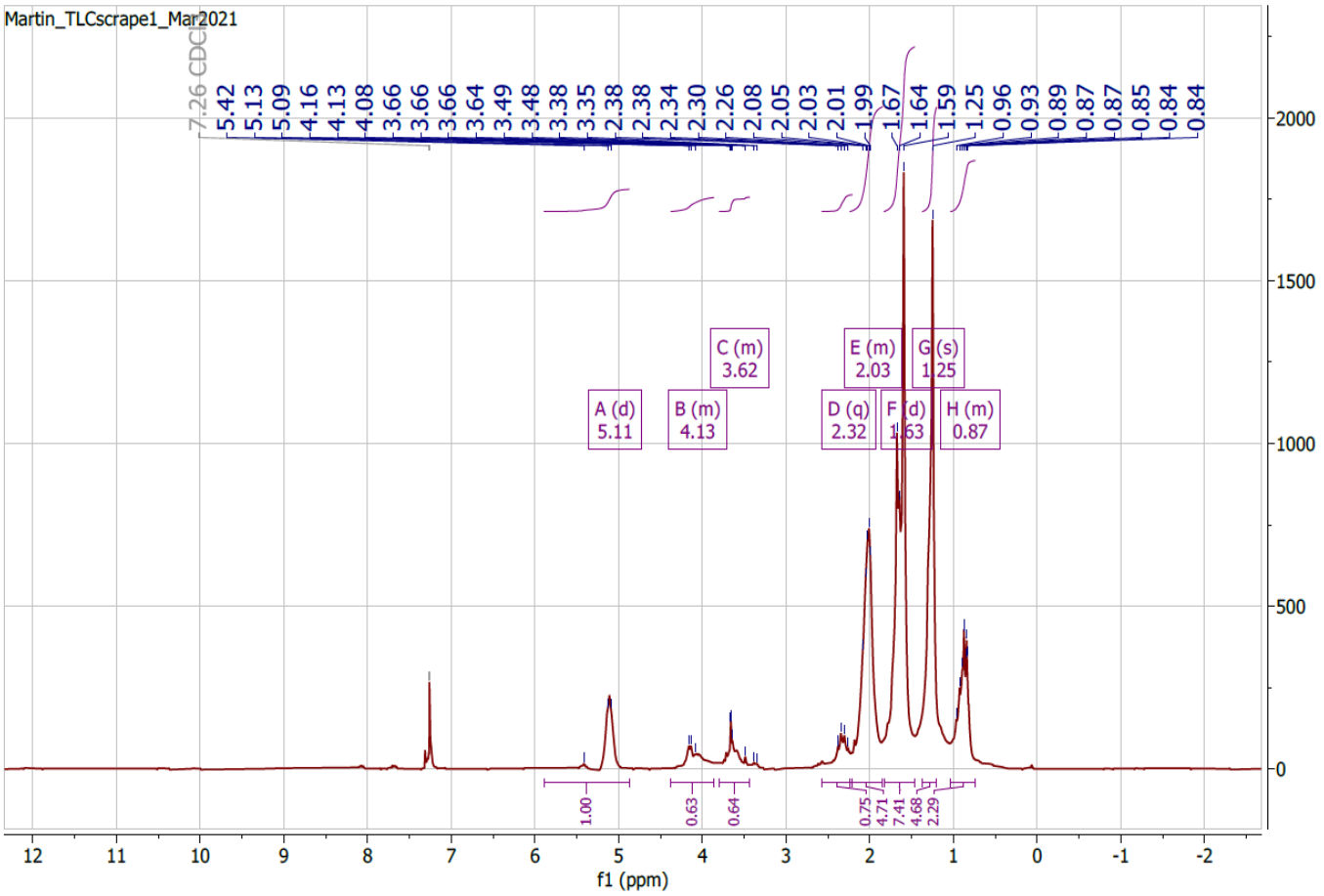


Figure 4.10: Annotated peaks of the ^{1}H-NMR spectrum of the semi-purified Spec1 fraction, scrapped from region 1 on the TLC plates (Figure 4.9). Information on the major peaks has been indicated in the purple boxes. A is a doublet (5.11ppm), B a multiplet (4.13ppm), C also a multiplet (3.62ppm), D is a quartet (2.32ppm), E is a multiplet (2.03ppm), F is a doublet (1.63ppm), G is a singlet (1.25ppm) and H is a multiplet (0.87ppm).

The ^{1}H-NMR enlarged areas of Spec1 (Figures 4.11 and 4.12) show the peaks more clearly. As this was a semi-purified sample, the individual peaks could be compared to those of protons on the carotenoid, staphyloxanthin and its precursors.

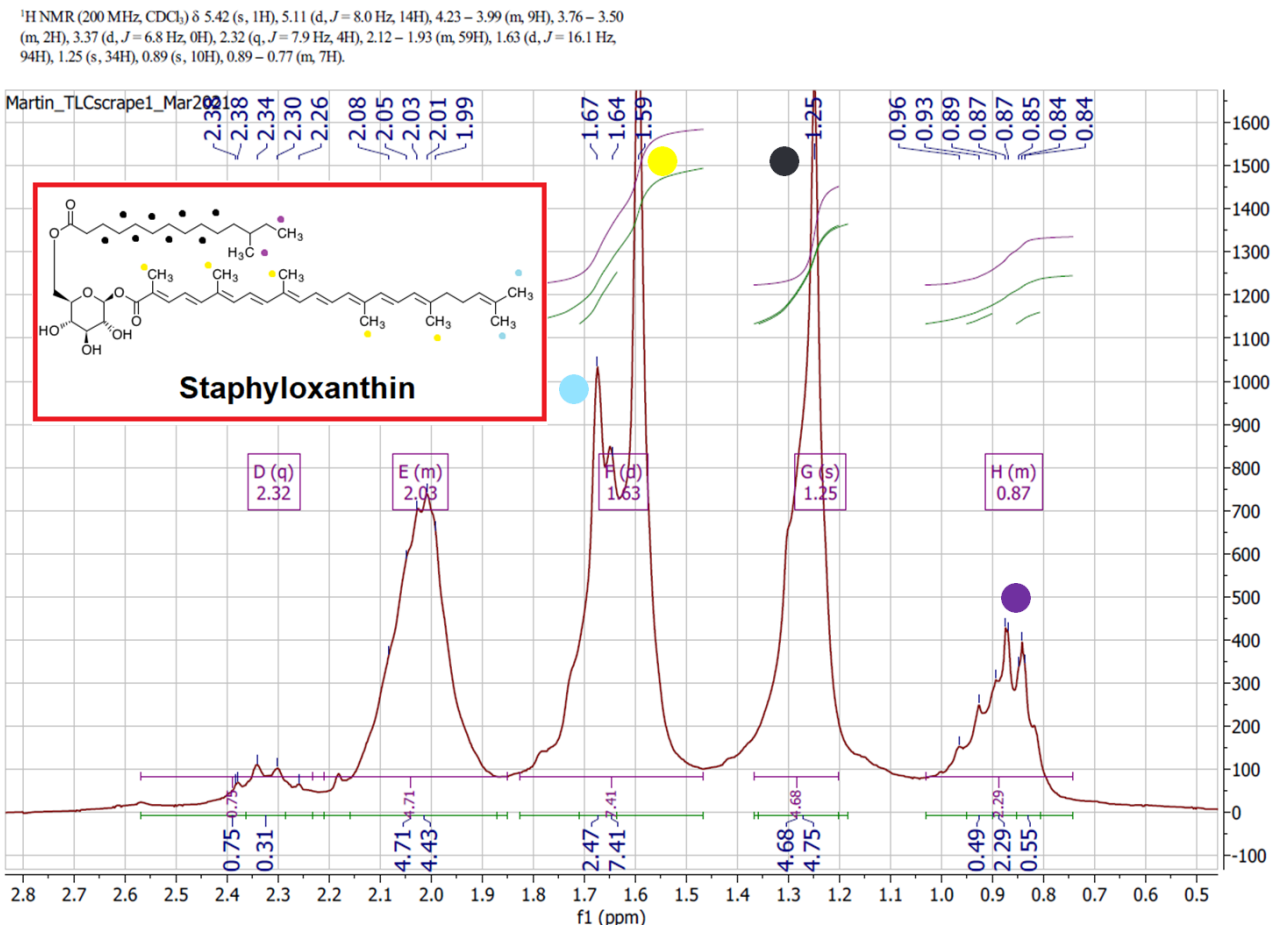


Figure 4.11: ^{1}H-NMR spectrum of Spec1 semi-purified fraction expanded between 0.5-2.8 ppm with inset staphyloxanthin structure showing corresponding hydrogens and their chemical shifts.

The ^{1}H-NMR peaks were determined to represent the different hydrogens on the staphyloxanthin and its precursor's structures. The 18 hydrogens on the saturated chain were determined to represent the large singlet at 1.25 ppm while the 15 hydrogens of the methyl groups on the unsaturated chain are responsible for the second largest singlet at 1.63 ppm.

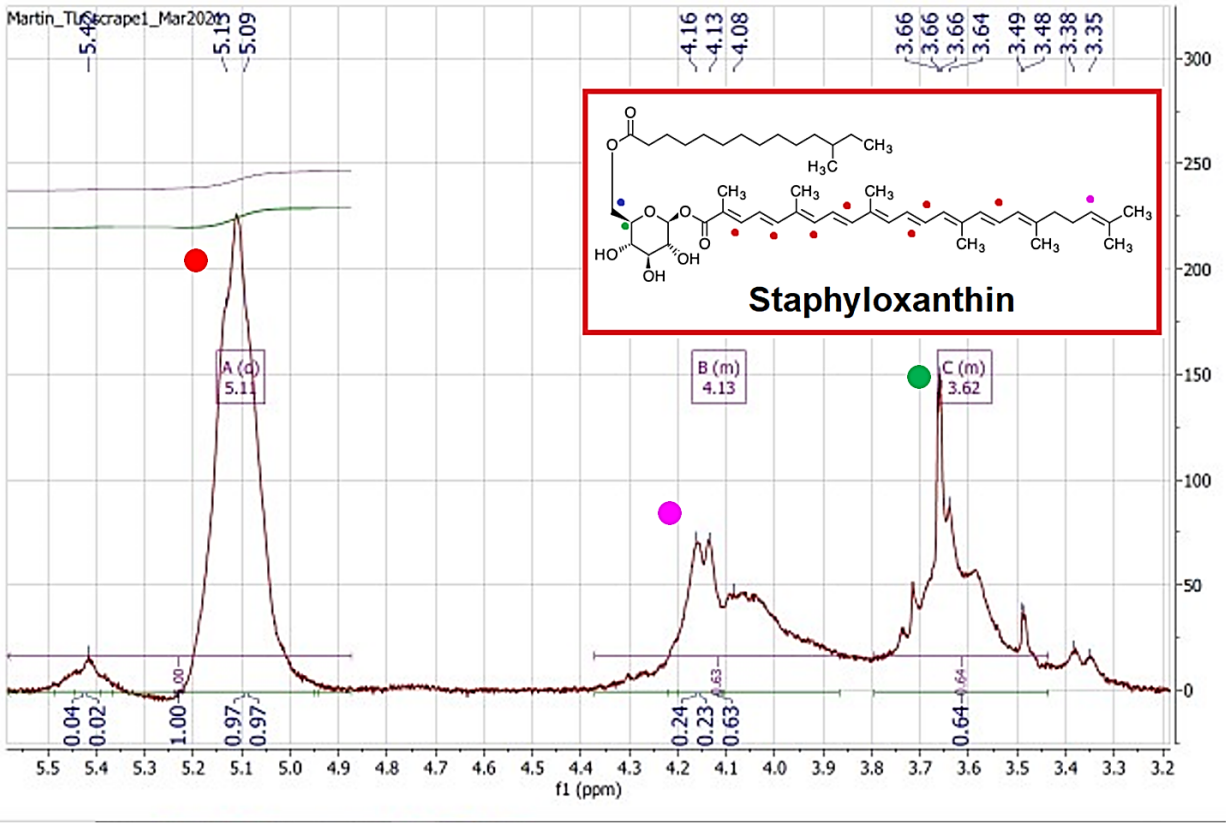


Figure 4.12: ^{1}H-NMR spectrum in Figure 4.10 expanded between 3.2-5.5 ppm with inset of staphyloxanthin structure showing corresponding hydrogens.

The seven hydrogens on the unsaturated chain were determined to represent the large doublet downfield at 5.11 ppm while the doublet at around 4.13 ppm could be from the hydrogens closer to the end of the chain.

The ^{1}H-NMR of Spec1 was compared to the ^{1}H-NMR analysis for staphyloxanthin carried out by Baretto and Voolta (Figure 4.13, 2018). In Figure 4.13, the proton NMR spectrum of staphyloxanthin shows the protons on the -OH groups at ppm(s) 4.97, 4.83 and 4.38 ppm on carbons C18, C19 and C20 respectively (Baretto and Voolta 2018). None of these peaks were observed in the spectra obtained in the current study. While the peak at 3.62 ppm indicated by the green dot in Figure 4.13 corresponds to the singlet at C17 in Figure 4.15, C21-H and C25 hydrogens at 6.45 and 7.28 ppm respectively (Figure 4.13) were not observed. The ^{1}H-NMR results were therefore not conclusive as some of the protons signals found in the literature were not observed. Furthermore the sugar part of the staphyloxanthin structure could not be identified in the spectra. This suggests that the structure of Spec1 is not staphyloxanthin but it could possibly be two long chain precursors of it, as will be discussed below. LC-MS and GC-MS analyses of the same sample were then carried out.

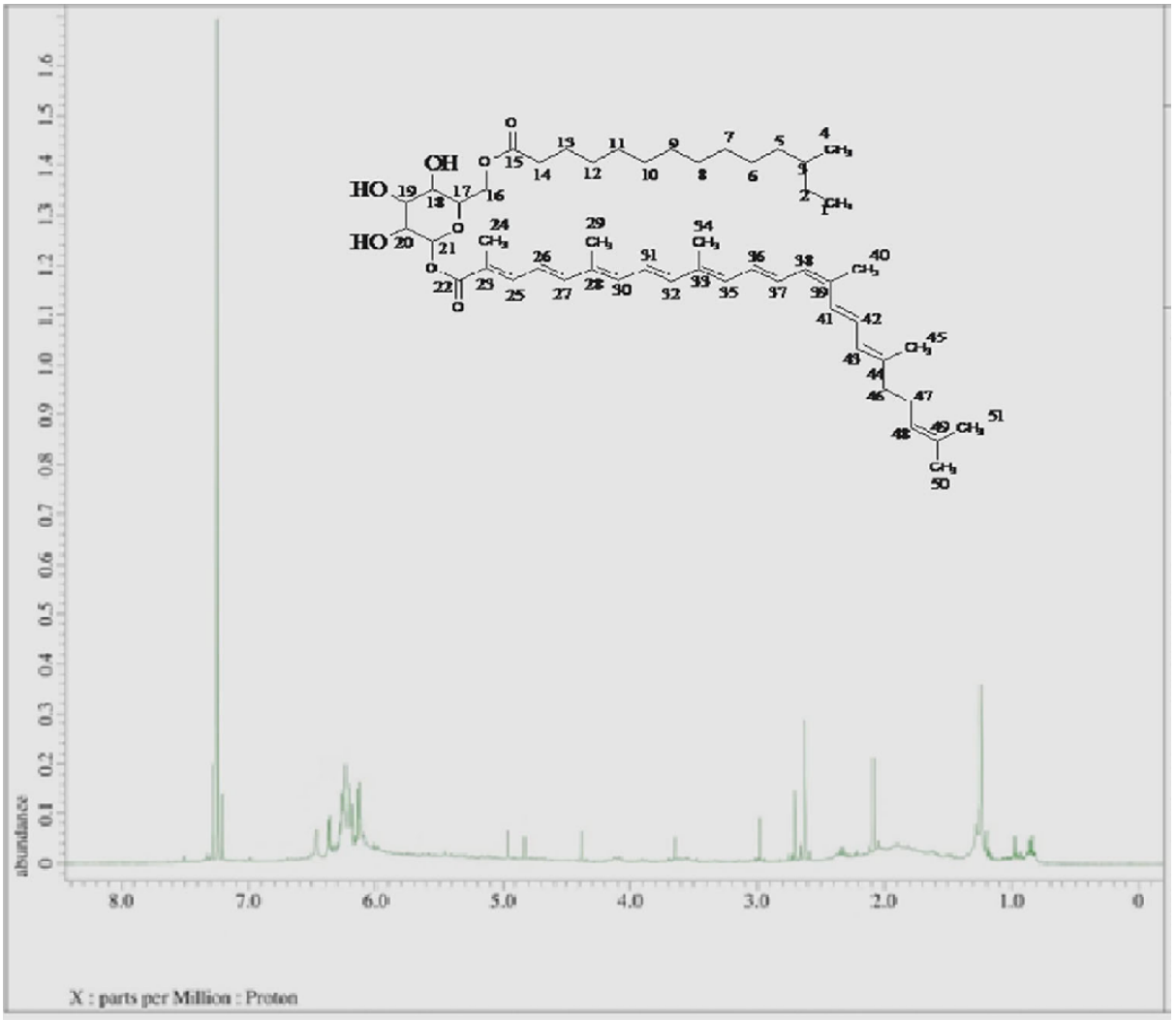


Figure 4.13: ^{1}H-NMR spectrum of the extracted staphyloxanthin pigment from *Staphylococcus gallinarum* isolated as the gut microflora bacterium of the insect *Bombyx mori* by Barretto and Voolta (2018).

4.4.3 LC-MS

Liquid chromatography mass spectrometry (LC-MS) was used to determine the presence of staphyloxanthin or its precursors by their MS and absorption spectra (Figure 4.14, Pelz et al., 2005). The absorption maxima for staphyloxanthin are at 463 nm and 490 nm according to Pelz et al. (2005).

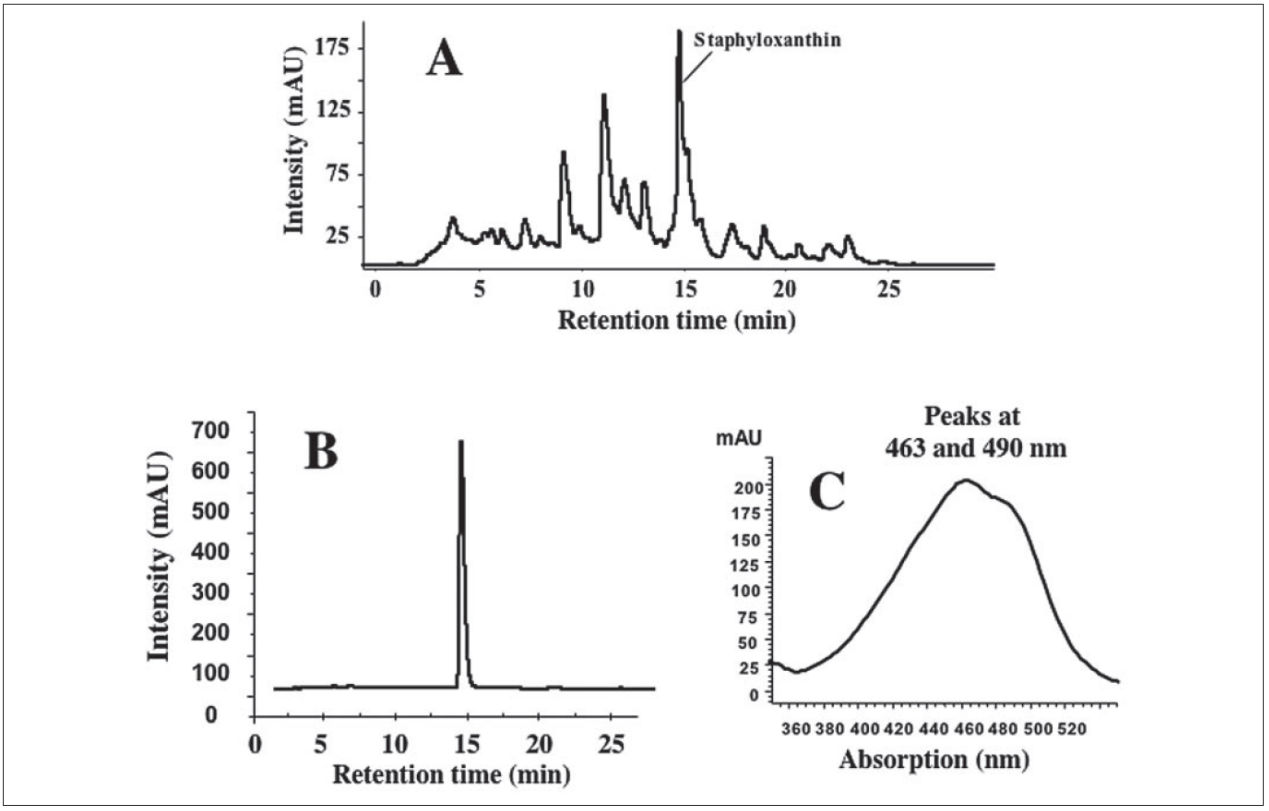


Figure 4.14: (A) HPLC-UV analysis of a crude extract of *S. carnosus*. (B) HPLC-UV analysis of purified staphyloxanthin used for NMR analysis. (C) Absorption spectrum of purified staphyloxanthin with the peaks at 463 and 490 nm (Pelz et al., 2005).

In the current study, acetonitrile was substituted for the acetone previously used by Pelz et al. (2005). This was because acetone was considered to be too corrosive for the plastic piping in the HPLC instrument used for this analysis. Acetonitrile was also found previously to be a viable substitute for acetone particularly when used in HPLC analysis with water (Funari et al., 2015).

In Figure 4.15 it can be seen that between 15 and 16 min (* on the Spec1 semi-purified fraction's chromatogram), the susceptible extract showed small peaks that didn't appear on the resistant extract. The peaks for staphyloxanthin were also observed at around 15 min at 400, 450 and 463 nm by Pelz et al. (2005). In the resistant HPLC PDA spectrum, there were peaks at 28.3 and 28.9 min that were not observed on the susceptible spectra (*).

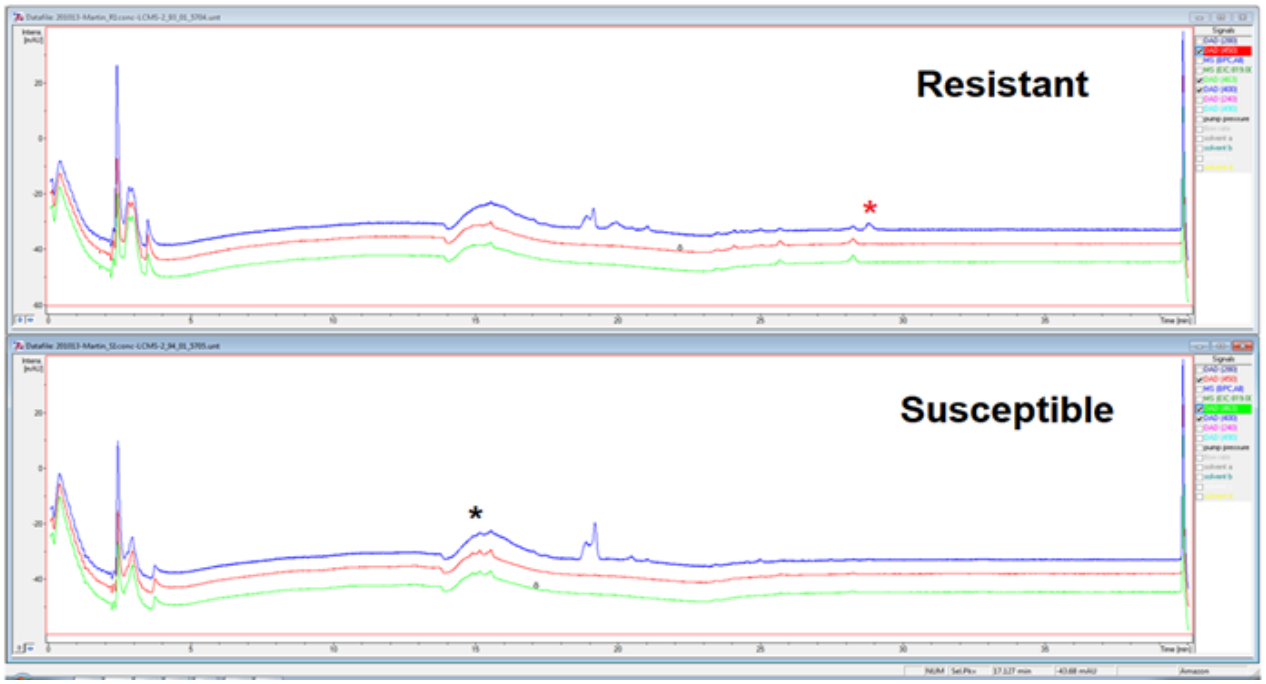


Figure 4.15: LC-MS spectra for resistant and susceptible crude extracts focusing on ranges of 400-463nm according to Pelz et al. (2005). The PDA (photodiode array) was set to different wavelengths shown by the different coloured spectral lines. The navy blue line was set at 400 nm, the red line at 450 nm and the light green line at 463 nm (Pelz et al., 2005).

Considering the similarities in the solvent systems used, the peaks seen for the susceptible spectra at 15 min could be those of staphyloxanthin or similar other compounds. The unique peaks observed for the resistant sample confirm observations in the first part of the experiment which showed that the resistant strain is producing new metabolites not present in the original susceptible strain.

The mass spectra results which were compared to MS data from Pelz et al. (2005) in Figure 4.16, showed the presence of a small quantity of a compound, maybe staphyloxanthin, in the susceptible sample with a 801.2 m/z mass. This was absent from the resistant strain which was previously found to not produce staphyloxanthin. Compared to the results from Pelz et al. (2005) there were no peaks for diaponeurosporene detected in both analyses. It is possible that other carotenoid pigments were present as staphylococcus bacteria produce a number of different carotenoid pigments (Marshall and Willmoth, 1985).

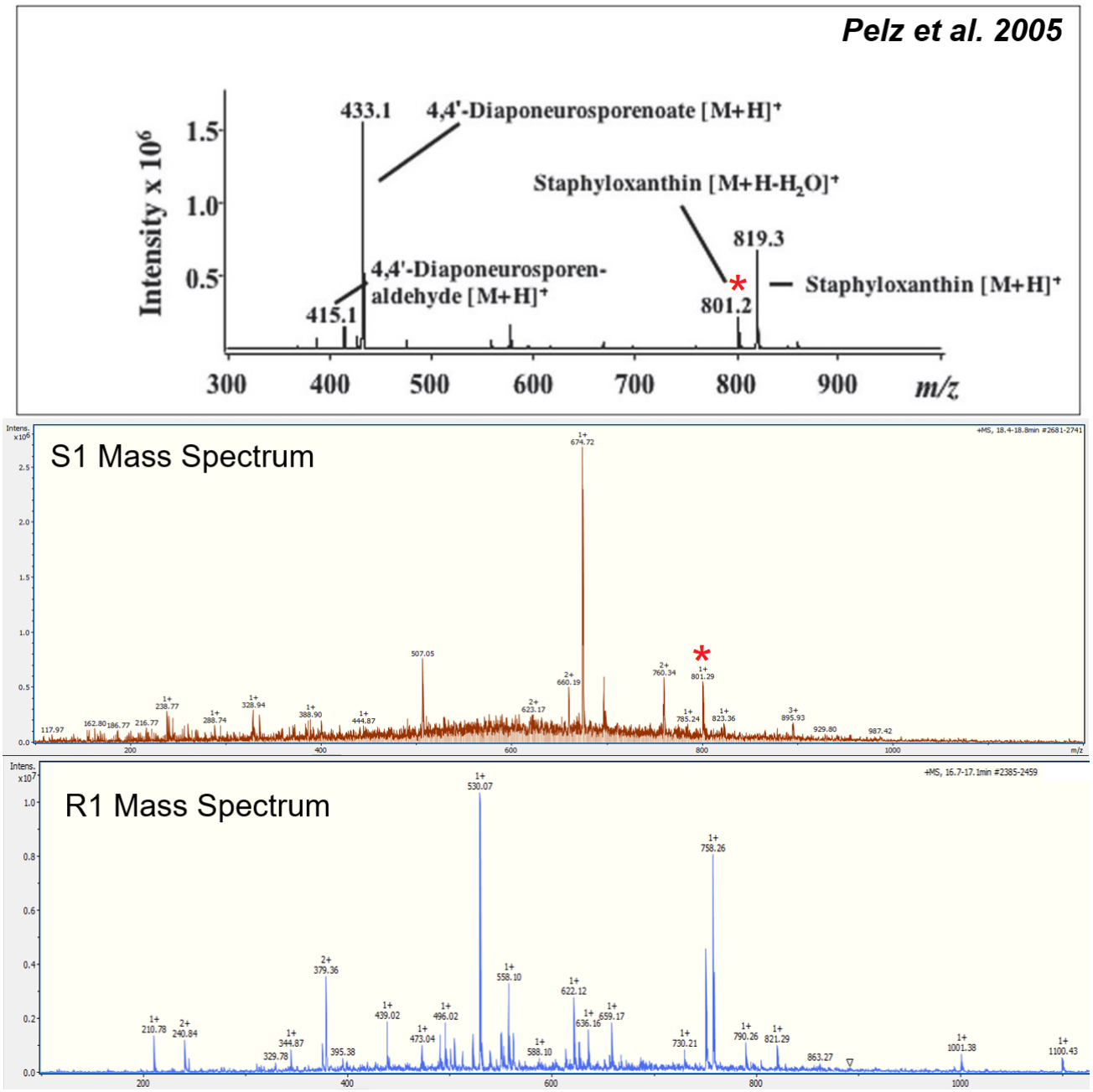


Figure 4.16: MS spectrum for staphylococcal carotenoid extracts from Pelz et al. (2005) compared to susceptible extract (S1) mass spectrum and resistant extract (R1) mass spectrum. The red star (*) at 801.2 m/z shows that staphyloxanthin could be present in the susceptible extract but not in the resistant extract.

4.4.4 GC-MS

After the analyses by ^{1}H-NMR and LC-MS to determine if staphyloxanthin is present in the methanolic extract's Spec 1 semi-purified fraction, it was also analysed by GC-MS. Barretto and Vootla (2015) also used GC-MS to verify their LC-MS and NMR data. The GC-MS chromatogram of the Spec1 semi-purified fraction showed a single high concentration peak at 43.96 min. (Figure 4.17)

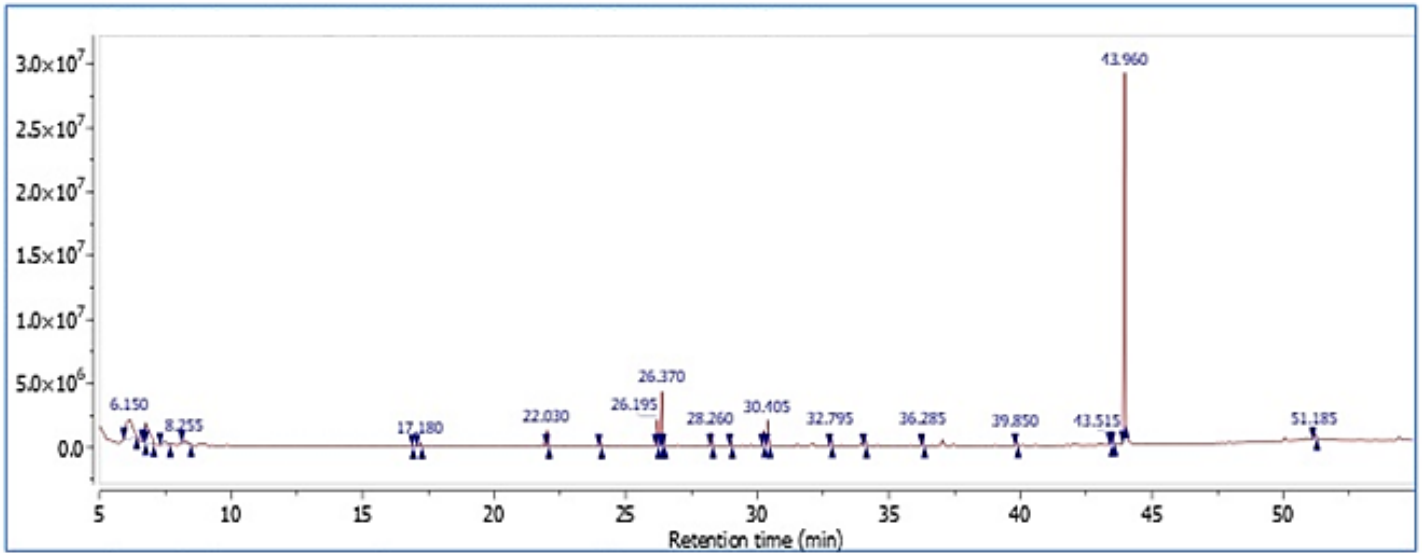


Figure 4.17: GC-MS chromatogram of the methanolic extract of the Spec1 fraction from the susceptible culture.

The prominent peak at 43.96 min in Figure 4.17 corresponded to some degree with squalene, a yellow coloured pigment, with a similarity of 59% according to the NIST library. Squalene is an unsaturated hydrocarbon chain with the same number of carbons (24) as the terpenoid carotenoid chain on the staphyloxanthin structure. The mass spectra for squalene and the Spec1 semi-purified compound are compared in Figure 4.18.

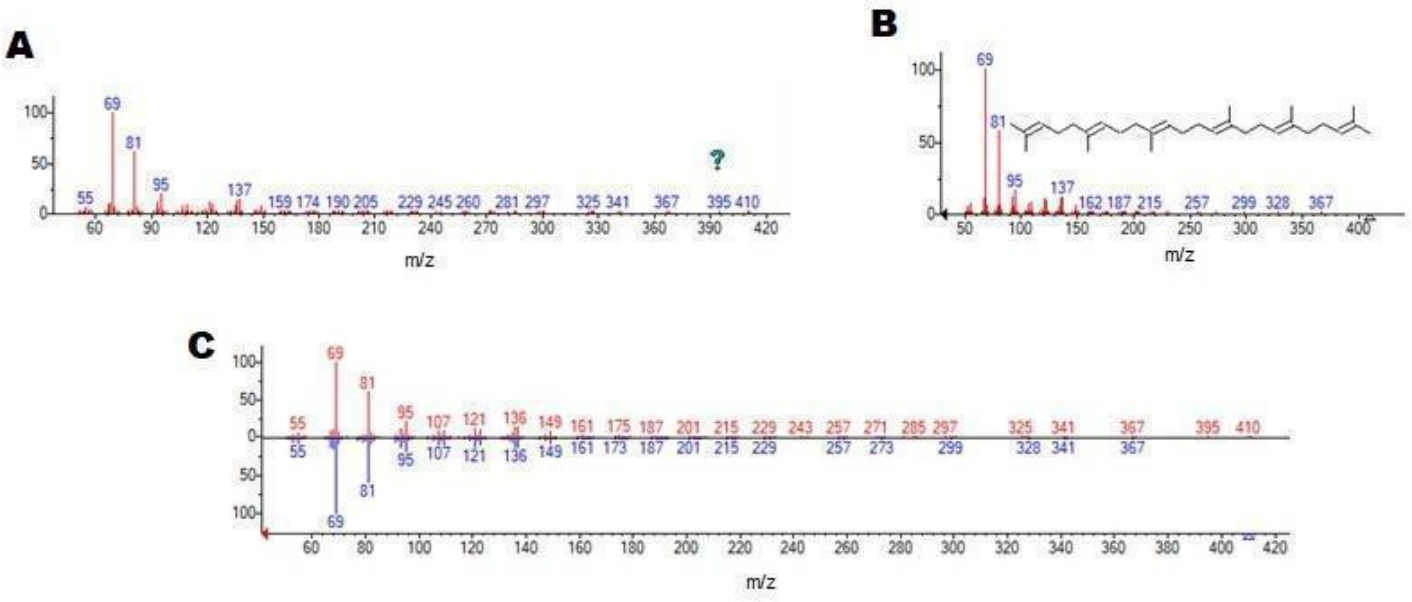


Figure 4.18: GC-MS results for the Spec1 fraction showing (A) the MS spectrum for the compound eluting at 43.96 min (B) the reference spectrum for squalene which is remarkably similar to staphyloxanthin's one long chain sidechain and (C) a side-by-side comparison of the spectra on the NIST database with the blue spectrum being the reference MS for squalene while the red spectrum (top) is the bacterial extract compound analysed.

The second most prominent peak in the GC-MS chromatogram was at 26.37 min (Figure 4.18) and was also analysed using the NIST library reference. This corresponded partially to tetradecanoic acid (colourless, similarity 42.5%), a C15 carbon chain very similar to the C15 esterified alkane chain on the staphyloxanthin structure (Figure 4.19).

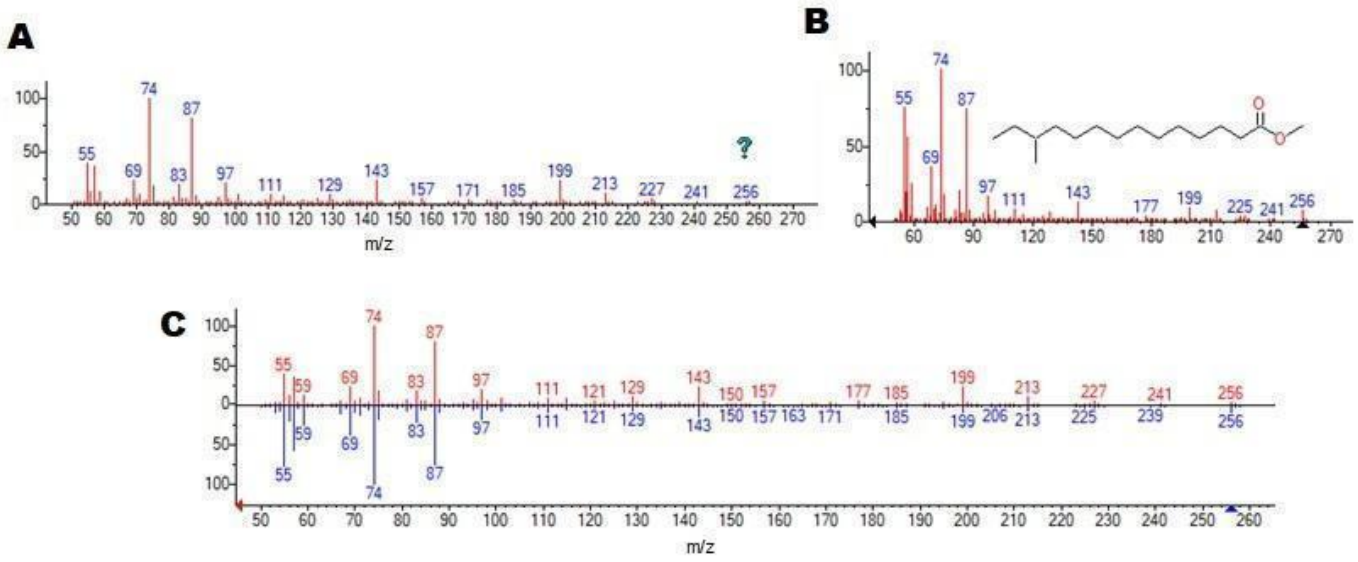


Figure 4.19: GC-MS results for methanolic extract showing (A) the MS spectrum for the compound eluting at 26.37 min (B) the reference spectrum for tetradecanoic acid and (C) a side-by-side comparison of the spectra with the blue MS spectrum being the reference MS for tetradecanoic acid while the red spectrum (top) is the bacterial extract compound analysed.

The GC-MS results showed that the two precursor side chains of staphyloxanthin, having similarity to squalene which has a yellow colour and tetradecanoic acid, seems to be present in the susceptible bacterial extract. The mass spectrum from the LC-MS analysis showed a fragment of 801 m/z in the crude extract (Figure 4.16) which did not feature in the GC-MS analysis of the purified extract. GC-MS analysis may not have shown the presence of the staphyloxanthin structure as the compound is not volatile enough, however, it seems as though the volatile precursors or similar compounds could be present in the susceptible bacterial extract.

In their analysis of *S. xylosus* carotenoids, Seel et al. (2020) noted that about 85% of the carotenoids that they detected were 'staphyloxanthin or staphyloxanthin-like derivatives'. They also noted that their mass spectrum analyses showed the staphyloxanthin-like compounds they detected differed from staphyloxanthin at the esterified fatty acid at the carbohydrate residue. The GC-MS results obtained in the current study seem to correlate with this finding as the esterified region on the fatty acid and carbohydrate residue were not detected in either the ^{1}H-NMR (nor the GC-MS, but staphyloxanthin is not volatile) analyses. The yellow colour observed in the susceptible bacteria could possibly be ascribed to a metabolite that is very similar to squalene.

Chapter 5: Conclusion and Future Prospects

5.1 Conclusions of the study

The first objective of the study to create an antibiotic resistant bacterium from a previously susceptible strain was achieved. A comparative metabolomic analysis between an antibiotic resistant bacteria culture and its antibiotic susceptible wild-type strain was also successfully carried out. During this comparative analysis, significant chemical differences in the profiles of both strains were observed and characterized.

Antibiotic resistance tests were carried out using the common beta-lactam antibiotics, methicillin and penicillin and confirmed by using the Kirby-Bauer susceptibility test (Beimer, 1973). The final objectives of the experiment, which included creating an antibiotic resistant strain of *S. xylosus* from a previously susceptible one, performing a comparative metabolomic analysis and isolating a potential biomarker from one of the strains were also achieved. The metabolic profiles of both strains were analysed by ^{1}H-NMR, LC-MS and GC-MS analyses. The results obtained from this study demonstrated the possibility of identifying the chemical differences between an initially antibiotic susceptible wild-type and its consequently antibiotic resistant strain.

The antibiotic resistant strain was created by subjecting the culture to starvation stress. This resulted in the starved culture developing resistance at antibiotic concentrations that the wild-type was previously shown to be susceptible to. It was deduced that the resistance may have been caused by a reduction in metabolic activity as non-replicating bacteria in the culture emerged with an inherent indifference to drugs. It was observed that the resistant strain was able to retain its insensitivity to antibiotics when re-cultured in new broth. This suggests that the resistant phenotype may have been inherited by a new generation not exposed to the same stresses that induced antibiotic resistance in the starved strain. It is also possible that certain quorum sensing activating molecules were carried over during the re-culturing procedure which elicited the same response on the new colonies. The possibility of genetically inherited induced mutations was not investigated in this study.

For the first part of the study, only one method of analysis (^{1}H-NMR) was carried out on six replicates of each strain. The ^{1}H-NMR results showed a high level of consistency and reproducibility. The susceptible replicates were all susceptible to the antibiotics and the resistant ones were all resistant. The spectra from all the resistant strain showed unique proton signals that were not observed in the susceptible data. The PCA analysis confirmed that there were significant chemical differences between the wild-type antibiotic susceptible culture and its antibiotic resistant strain.

The second part of the study was based on characterizing a bright yellow-orange pigment which was observed in the susceptible strain but not the resistant strain. While it was hypothesized that there would be differences in the resistant strain rather than the susceptible strain, the observation could still prove to be of value in future studies.

The focus on this yellow-orange pigment which could be produced by staphyloxanthin or its precursors, came after two Petri dishes were left out on a lab bench for a few days. The susceptible culture turned bright orange while the resistant remained white. The staphyloxanthin was then extracted from the bacteria cultures by washing the cultures in methanol and separated by centrifugation. This was done for both resistant and susceptible cultures before both were analyzed by ^{1}H-NMR and LC-MS. Proton NMR results showed the presence of both the C15 fatty acid chain and the long unsaturated carbon chain on the susceptible spectra, but the sugar ring on the staphyloxanthin structure was not detected. The LC-MS results indicated the possible presence of a small concentration of staphyloxanthin in the susceptible strain but not the resistant strain.

The amount of pigment was increased for analysis by upscaling the production of *S. xylosus* of the susceptible culture which were then analysed by GC-MS with the mass spectra being compared to spectra on the NIST library. The results of the GC-MS analysis reflected the previous ^{1}H-NMR results in which the two long carbon chains (similar in structure to squalene and tetradecanoic acid) were observed of staphyloxanthin. The NIST library does not contain staphyloxanthin, so it's still not sure that the compound is produced by the susceptible culture.

It was also noted that there were certain metabolites being produced by the susceptible strain and not by the resistant strain, and vice versa. This shows that as the susceptible

strain became resistant to antibiotics, there were certain metabolites that were no longer produced while new metabolites were in turn synthesised.

The objective of the study was to find new targets or biomarkers in the antibiotic bacteria, however the antibiotic susceptible compound was found to produce staphyloxanthin while the resistant one was not found to produce the pigment. While inhibiting the production of staphyloxanthin won't directly stop antibiotic resistance, it could be an important step in limiting the development of resistance. As discussed, staphyloxanthin helps to protect bacteria from ultraviolet radiation and radical oxidative species as well as neutrophils in the innate immune system. Without staphyloxanthin, the bacteria's survival period could be limited thus reducing the time that the bacteria takes to grow and adapt to an environment. This would consequently reduce the rate of acquiring resistance.

5.2 Future prospects in continuing the study

It is recommended that in continuing this study, more methods of inducing antibiotic resistance be investigated, including longer periods of starvation, starving of specific essential nutrients (e.g., glucose), increasing antibiotic stress, etc. Since mutations are a driving factor of adaptive antibiotic resistance (Woodford and Ellington, 2007), further studies could also be carried out in observing the rates of mutagenesis caused by the different stress factors and how they drive adaptive antibiotic resistance (Schroder et al., 2018).

In addition, it is recommended that genomic analyses be carried out in further studies comparing the differences between the susceptible and resistant bacterial cultures. It would be interesting to observe which genes are being activated and which are deactivated, which protein products are being up-regulated and which are down-regulated. These would all serve to give more comparative information between the resistant mutant strains from their susceptible wild-type strains.

Furthermore, the combination of various synergistic plant antimicrobials could be assessed for its activity against the development of mutations which cause antibiotic resistance. It was noted that a large amount of antibiotic resistance develops by adaptive mutation during the course of antibiotic treatment (Smith and Romesberg, 2007). This was

particularly noted for *Mycobacterium tuberculosis* which develops resistance by adaptive resistance to antibiotics by mutating during treatment (Boshoff et al., 2003). In this case, the comparative approach proposed by this study may also be useful in determining which combined synergistic combinations are able to restrict the antibiotic resistance mechanisms while limiting bacterial mutation. Observations could be noted in the changes in biochemistry (mutations) during antimicrobial therapy. Having a metabolic profile of both resistant mutants and susceptible wild-type strains, it might be possible to accurately observe which synergistic plant combinations are active with lower mutation rates during antibiotic treatment of the bacteria.

Co-amoxiclav, an antibiotic combination of amoxicillin and clavulanate, is an example of the sort of synergistic combinational treatments that a future study could investigate. Clavulanate is a β-lactamse inhibitor which protects β-lactam antibiotics from β-lactamses produced by antibiotic resistant bacteria. Combining the β-lactam antibiotic amoxicillin with clavulanic acid allowed for the treatment of previously β-lactam resistant bacteria species such as *Klebsiella* and *Prevotella* (Ball et al., 1997). The model of comparative analysis proposed in this study could be used to identify more non-essential mechanisms that enable antibiotic resistance but are not essential for bacterial growth. By targeting these non-essential mechanisms, the bacteria will experience less stress thus reducing its adaptability and mutation during the course of antibiotic treatment (Annunziato, 2019).

While plant chemotherapeutics are already being used to confront antibiotic resistance in the clinical setting, there is a greater focus on antibiotic stewardship and finding new antibiotics. In a changing world, the problems we are experiencing are becoming less natural and more a result of human activity. This is a theory proposed by German sociologist Ulrich Beck who devised the concept of the *Risk Society* (Beck, 1992). According to Beck's Risk Society concept, the modern industrialized society is shaped by risks and extreme crises of its own making. These may include climate change, COVID 19 and the looming antibiotic resistance crisis. Beck (1992) defined these 'risks of modernisation' as "irreversible threats to the life of plants, animals and human beings". Following the tremendous effects that the COVID-19 pandemic has had on the world, sociologists, Lupton and Willis (2021) proposed that we are currently living in a 'COVID society' based on their understanding of Beck's risk society model and their work on the COVID-19 pandemic (Lupton and Willis, 2021).

The COVID-19 pandemic was a result of modernization and was driven by human factors such as the globalization of travel which meant different people and different animals which would previously have never met when interacting in close proximity at a higher frequency. The antibiotic crisis is also a man-made problem because it was created by isolating antibacterials which only existed in limited amounts and mass producing them. This exposed many bacteria to pressures they previously never experienced thus they became more resilient than they had been before. The antibiotic resistance crisis goes beyond the scope of natural phenomena studied in science. The COVID-19 pandemic gives an example of the tremendous damage and disruption that a sudden health crisis could cause to various aspects of the world such as the economy, healthcare systems. The COVID-19 pandemic showed the difficulty that scientists, health professionals, policy makers as well as the general public had in responding to a fast-changing and fast spreading health crisis. These difficulties caused hesitations and misjudgements which resulted in significant loss of life.

The antibiotic resistant crisis is already seen as being as big a threat to humanity as climate change (Torjesen, 2013). The COVID-19 pandemic could be seen as an opportunity to observe what a serious antibiotic resistance crisis where infectious bacteria spreading amongst large swathes of the population without any immediate cure can cause on the public as well as the scientists and healthcare professionals expected to respond to it. Understanding Beck's risk society model, it might be possible to understand the human effect of the Covid-19 pandemic and possibly use this to pre-empt a wide-scale antibiotic resistance crisis.

There are also many factors affecting and worsening the antibiotic resistance crisis that go beyond the laboratory setting. These include antibiotic stewardship, health policy, education etc. According to Pietrocola et al. (2021) the COVID-19 pandemic showed the deficiency in the current education system in educating science students on how to deal with manufactured risks. This has exposed a collective lacking in society’s ability to find and implement rational solutions in the face of uncertain situations such as a global pandemic. As seen with the COVID-19 pandemic, once people become anxious, they demand explanations and decisions on imponderable and often immeasurable factors. The scale and rate of such risks often means that decisions-making cannot rely on previous experiences nor resorting to experts (Pietrocola et al., 2021).

As the calls for new and alternative treatments against antibiotic resistance grow louder, it is expected that the need for new methods of characterization for these alternative treatments will rise.

While the proposed comparative analysis approach in this study may be improved by adding newer more thorough methods of data acquisition, its fullest potential may only be realized if computer models could be designed for the process, based on the trends in data acquired. Computer modelling could mean that the bulk of analytical work could be carried out on computer systems outside the wet laboratory setting greatly reducing the time and resources required to run the tests. Indeed, one of the greatest obstacles to pharmaceutical drug discovery is the tedious laboratory work in screening numerous treatment options.

Spectral information acquired from different analyses on the wild-type and mutant metabolic profiles, the chemical changes when cultures are exposed to different combinations of antimicrobial compounds, the rate and trends in the induced antibiotic resistant mutation and the combined synergistic effects of the plant antimicrobials against the resistant mutants could be compiled to form advanced computer models which could simulate more trials so that even more analyses could be carried. Computer models predicting the best synergistic plant antimicrobial combinations for specific mutations causing induced antibiotic resistant bacteria could be the future path for incorporating effective plant-based chemotherapeutic treatments in treating problematic antibiotic resistant pathogens.

It may be possible to use the data obtained from analysing the biochemical and pharmacological activity of the effective synergistic plant combinations to design an effective synthetic drug. It has already been shown by Jenssen et al. (2008) that it is possible to build detailed predictive models to test the effectiveness of synthetic drugs, even the small sets of structurally different peptides and that such models can convert primary structure information into PLS (partial least square) models. These could enable the modelling of structurally diverse antimicrobial peptides. Jenssen et al. (2008) have also developed a predictive model that is based on a limited selection of peptides from two distinctly related peptide libraries and successfully showed its potential in accurately

predicting the activity of a section that had been intentionally excluded part of the library. This proves that computer modelling could have far-reaching effects in predicting the effects of complex biochemical interactions of antimicrobial agents.

Once the antimicrobial activity is well understood and compiled into a computer model, the design of a synthetic treatment mimicking the synergistic antimicrobial activity might be more probable.

The proposed direction in the study, namely the acquisition of more data on adaptive mutation, genetic expression of adaptive mutation and analysis of combined antibacterial interactions are the start of this greater concept of creating a significant repository of data that may become a reality for future generations. Such a repository could grow over the years to one day being incorporated into an advanced computer model from whence an ultimate and lasting solution to the antibiotic crisis could be found.

Antibiotic resistance is a natural phenomenon, as noted by Flemings (1929) and Aminov (2007, 2010). However, the proliferation of this problem into a global crisis with potentially pandemic proportions is largely a man-made crisis. As previously discussed by sociologists like Beck (1992) such risks are an inevitable product of the modern world. However, another inevitable aspect of the modern world is the constant drive for innovation and the pursuit of knowledge.

Like the bacteria adapting to antibiotics, humans must now adapt to bacteria resistant to antibiotics. As bleak as the situation might seem, there is still a lot of hope. With continued cooperation between the various fields of science, health science and even those fields outside the realm of the hard sciences, we can find new ways to perceive the problem and new solutions. With an open mind and a commitment to never stop learning, the possibilities are endless.

References

Abreu AC., McBain, AJ., Simñoes, M. 2012. Plants as sources of new antimicrobials and resistance-modifying agents. RSC Natural Products Report. 29, 1007–1021

Allen, HK., Moe, LA., Rodbumrer, J., Gaarder, A., Handelsman, J. 2009, Functional metagenomics reveals diverse Lactamases in a remote Alaskan soil. The ISME Journal. 3, 243-251.

Aminov, RI. 2009. The role of antibiotics and antibiotic resistance in nature. Environmental Microbiology. 11(12), 2970-2988.

Aminov, RI. 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. Frontiers in Microbiology. 134, 1-7.

Aminov, RI., Mackie, RI. 2007. Evolution and ecology of antibiotic resistance genes. FEMS Microbiology Letters. 271, 147–161.

Asaad, AA., Qureshi, MA., Hasan, SM. 2016. Clinical significance of coagulase-negative staphylococci isolates from nosocomial bloodstream infections, Infectious Diseases, 48:5, 356-360

Bagge, N., Schuster, M., Hentzer, M., Ciofu, O., Givskov, M., Greenberg, EP., Hoiby, N. 2004. *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and β-Lactamase and alginate production. Antimicrobial Agents Chemotherapeutics. 48, 1175-1187.

Ball, AR., Casadei, G., Samosorn, S., Bremner, JB., Ausubel, FM., Moy, TI. 2006. Conjugating berberine to a multidrug resistance pump inhibitor creates an effective antimicrobial. ACS Chemical Biology. 1, 594–600.

Barber, M. 1948. Infection by penicillin resistant Staphylococci. Lancet. 2, 641-644.

Beck, U., 1992. Modern society as a risk society. The Culture and Power of Knowledge. Inquiries into Contemporary Societies, pp.199-214.

Bennett, PM. 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. British Journal of Pharmacology. 153, S347–S357

Bosch, F., Rosich, L. 2008. The Contributions of Paul Ehrlich to Pharmacology. Pharmacology. 82, 171–179.

Boshoff, HI., Reed, MB., Barry, CE., Mizrahi, V. 2003. DnaE2 polymerase contributes to *in vivo* survival and the emergence of drug resistance in *Mycobacterium tuberculosis*. Cell. 113, 183–193.

Broadhurst, DI., Kell, DB. 2006. Metabolomics. 2:171–196.

Biemer, JJ. 1973. Antimicrobial Susceptibility Testing by the Kirby-Bauer Disc Diffusion Method. Annals of Clinical Laboratory Science. 3(2), 135-140

Brötz-Oesterhelt, H., Brunner, NA. 2008. How many modes of action should an antibiotic have?. Current Opinion in Pharmacology. 8(5), 564-573.

Centers for Disease Control and Prevention. Antibiotic resistance threats in the United States, 2013. Available at: https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf. [Accessed April 27, 2021]

Chain, E., Florey, HW., Gardner, AD., et al. 1940. Penicillin as a chemotherapeutic agent. Lancet. 24:226-23.

Clauditz, A., Resch, A., Wieland, K.P., Peschel, A. and Gӧtz, F., 2006. Staphyloxanthin plays a role in the fitness of Staphylococcus aureus and its ability to cope with oxidative stress. Infection and immunity, 74(8), 4950-4953.

Christensen, GD., Simpson, WA., Younger, JJ., Baddour, LM., Barrett, FF., Melton, DM., Beachey, EH. 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. Journal of Clinical Microbiology. 22, 996-1006.

Coker, MS., Forbes, LV., Plowman-Holmes, M., Murdoch, DR., Winterbourn, C.C., Kettle, AJ., 2018. Interactions of staphyloxanthin and enterobactin with myeloperoxidase and reactive chlorine species. Archives of biochemistry and biophysics, 646, 80-89.

Cook, M., Molto, E., Anderson, C. 1989. Fluorochrome labelling in Roman period skeletons from Dakhleh Oasis, Egypt. American Journal of Physical Anthropology. 80, 137-143.

Cooper, T., Shlaes, J. 2011. Fix the antibiotic pipeline. Nature. 472, 32.

Crofton, J., Mitchison, DA. 1948. Streptomicin resistance in pulmonary tuberculosis. British Medical Journal. 2, 1009-1015.

Cuperlovic-Culf, M., Barnett, DA., Culf, AS., Chute, I. 2010. Drug discovery today. 15, 610-621.

Cuirolo, A., Plata, K., Rosato, AE. 2009. Development of homogeneous expression of resistance in methicillin-resistant *Staphylococcus aureus* clinical strains is functionally associated with a β-lactam-mediated SOS response, Journal of Antimicrobial Chemotherapy, 64(1), 37–45.

Cusini A, Rampini SK, Bansal V, Ledergerber B, Kuster SP, Ruef C, et al. 2010. Different Patterns of Inappropriate Antimicrobial Use in Surgical and Medical Units at a Tertiary Care Hospital in Switzerland: A Prevalence Survey. PLoS ONE. 5(11), e14011.

Datta, N., Kontomichalou, P. 1965. Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. Nature. 208, 239-41.

Davies, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science. 264, 375-82.

Davies, J., Spiegelman, GB., Grace Yim, G. 2006. The world of subinhibitory antibiotic concentrations. Current Opinion in Microbiology. 9(5)445-453.

Davies, J, Davies, D. 2010 Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews. 74(3), 417-433.

Davies, J. 2012. Antibiotic discovery: then and now. Microbiology Today. 39(4), 200-203.

De Cock KM, Marston, B. 2005. The sound of one hand clapping: tuberculosis and antiretroviral therapy in Africa. American Journal of Respiratory Critical Care and Medicine. 172, 3-4.

Debbia, EA., Roveta, S., Schito, AM., Gualco, L., Marchese, A. 2001. Antibiotic persistence: the role of spontaneous DNA repair response. Microbial Drug Resistance. 7, 335-342.

Delcour, AH. 2009 Outer membrane permeability and antibiotic resistance 2009. Biochimica et biophysica acta. 1794, 808-16

Donegan, N.P., Manna, A.C., Tseng, C.W., Liu, G.Y. and Cheung, A.L., 2019. CspA regulation of Staphylococcus aureus carotenoid levels and σ^B activity is controlled by YjbH and Spx. *Molecular microbiology*, *112*(2), 532-551.

Dordet-Frisoni, E., Dorchies, G., De Araujo, C., Talon, R., Leroy. 2007. Genomic Diversity in *Staphylococcus xylosus.* Applied and Environmental Microbiology. 73(22). 7199–7209.

Dowling, A., O'Dwyer, J., Adley, CC. 2013. Alternatives to antibiotics: future trends. Formatex, 216-217.

Fairbrother, RW. 1956. Mixed staphylococcal infections. The development of penicillin-resistant strains. Lancet, i, 716.

Fischer, N., Neumann, P., Stark, H. 2015. Structure of the *E.coli* ribosome-EF-Tu complex at <3 Å resolution by CS-corrected cyro-EM. Nature. 520, 567-570.

Fisher JF, Meroueh SO, Mobashery, S. 2005., Bacterial resistance to beta-lactam antibiotics: compelling opportunism, compelling opportunity. Chemical reviews. 105, 395-424.

Fleming, A. 1929. On the antibacterial action of cultures of a Penicillum with special reference to their use in the isolation of *B. Influenzae*. British Journal of Experimental Pathology, 226-236.

Forsberg, KJ., Patel, S., Wencewicz, TA., Dantas, G. 2015. The Tetracycline Destructases: A Novel Family of Tetracycline-Inactivating Enzymes. Chemistry and Biology. 22, 888–897.

Funari, CS., Carneiro, RL., Khandagale, MM., Cavalheiro, AJ., Hilder, EF. 2015. Acetone as a greener alternative to acetonitrile in liquid chromatographic fingerprinting. Journal of Separation Science, 38, 1458–1465

Gandhi, NR., Moll, A., Sturm, AW., Pawinski, R., Govender, T., Lalloo, U., Zeller, K., Andrews, J., Friedland, G. 2006. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet. 368, 1575–80.

Gao, P., Davies, J. and Kao, R.Y.T., 2017. Dehydrosqualene desaturase as a novel target for anti-virulence therapy against Staphylococcus aureus. MBio, 8(5), pp.e01224-17.

Gibbon, S. 2008. Phytochemicals for bacterial resistance – Strengths, Weaknesses and Opportunities. Planta Medica. 74(6), 594-602.

Godfrey, HP., Bugrysheva, JV., Cabello FC. 2002. The role of the stringent response in the pathogenesis of bacterial infections, Trends in Microbiology, 10(8), 349-351

Gupta, K., Singh, S.P., Manhar, A.K., Saikia, D., Namsa, N.D., Konwar, B.K. and Mandal, M., 2019. Inhibition of Staphylococcus aureus and Pseudomonas aeruginosa biofilm and virulence by active fraction of Syzygium cumini (L.) Skeels leaf extract: in-vitro and in silico studies. *Indian journal of microbiology*, *59*(1), pp.13-21.

Halouska et al., 2013. Revisiting Protocols for the NMR Analysis of Bacterial Metabolomes. Journal of Integrated OMICS. 3(2), 120–137.

Hassanein, MM. 2019. Sulfonamides: far from obsolete International Journal of Contemporary Pediatrics, 6(6), 2740.

Hayek, SA., Gyawali. R., Ibrahim, SA. 2013. Antimicrobial Natural Products. Formatex, 910-921.

Hemaiswarya, S., Kruthiventi, AK., Doble, M. 2008. Synergism between natural products and antibiotics against infectious diseases. Phytomedicine. 15, 639–652.

Jenssen, H., Fjell, CD., Cherkasov, A., Hancock, REW. 2008. QSAR modeling and computer-aided design of antimicrobial peptides. Journal of Peptide Science. 14, 110-114.

Katzif, S., Lee, EH., Law, AB., Tzeng, YL., Shafer, WM. 2005. CspA regulates pigment production in Staphylococcus aureus through a SigB-dependent mechanism. Journal of Bacteriology. 187, 8181–8184

Kloos, WE., Schleifer, KH. 1986. Genus IV. *Staphylococcus*. Bergey's manual of systematic bacteriology, 1013– 1035.

Kumar, S., Varela, M. 2013. Molecular mechanisms of bacterial resistance to antimicrobial agents. Formatex, 522-534.

Lee Ligon, B. 2004. Penicillin: Its Discovery and Early Development. Seminars in Pediatric Infectious Diseases. 15, 52-57.

Lee, HH., Molla, MN., Cantor, CR., Collins, JJ. 2010. Bacterial charity work leads to population-wide resistance. Nature. 467, 82–85.

Levin, BR., Rosen, DE. 2006. Non-inherited antibiotic resistance. Nature Reviews Microbiology. 4, 556-562.

Levy, SB., Marshall, B. 2004. Antibacterial resistance worldwide: causes, challenges, responses; Nature Medicine Supplement. 10(2), S122-9.

Livermore, DM. 1995. beta-Lactamases in laboratory and clinical resistance. Clinical microbiology reviews. 8, 557-84.

Lupton, D., and Willis, K. 2021. The COVID-19 Crisis: Social Perspectives (1st ed.). Routledge.

Marshall and Wilmoth (1981) Pigments of Staphylococcus aureus, a Series of Triterpenoid Carotenoids, Journal of Bacteriology, 147(3), 900-913

Maenhaut-Michel, G., Shapiro, JA. 1994. The roles of starvation and selective substrates in the emergence of *araB-LacZ* fusion clones. The EMBO Journal. 13(21), 5229-5239.

Martinez, JL., Baquero, F. 2000. Mutation Frequencies and Antibiotic Resistance. Antimicrobial agents and Chemotherapy. 44(7), 1771–1777

McDermott, W. 1958. Microbial persistence. Yale Journal of Biology and Medicine. 30, 257-291.

Metris, A., Le Marc, Y., Elfwing, A., Ballagi, A., Baranyi, J. 2005. Modelling the variability of lag times and the first generation times of single cells of *E. coli*. International Journal of Food Microbiology. 100, 13-19.

Miller, C., Thomsen, EL., Gaggero, C., Mosseri, R., Ingmer, H., Cohen, SN. 2006. SOS response induction by β-lactams and bacterial defense against antibiotic lethality. Science. 305, 1629-1631.

Mukadi, YD., Maher, D., Harries, A. 2001. Tuberculosis case fatality rates in high HIV prevalence populations in sub-Saharan Africa. AIDS. 15**,** 143–52.

Nelson, ML., Dinardo, A., Hochberg, J., Armelagos, GJ. 2010. Brief communication: mass spectroscopic characterization of tetracycline in the skeletal remains of an ancient population from Sudanese Nubia 350–550 CE. American Journal of Physical Anthropology 143, 151–154.

Nguyen, D. et al. 2011. Active Starvation Responses Mediate Antibiotic Tolerance in Biofilms and Nutrient-Limited Bacteria. Science. 334, 982-986.

Nicholson JK., Lindon, JC., Holmes, E. 1999 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica. 29, 1181–1189.

Novick NP. 1963. Analysis by Transduction of Mutations affecting Penicillinase Formation in *Staphylococcus aureus*. Journal of General Microbiology, 33, 121-186

Olivier, A.C., Lemaire, S., Van Bambeke, F., Tulkens, P.M., Oldfield, E. 2009 Role of rsbU and Staphyloxanthin (SFX) in Intracellular Growth of Staphylococcus aureus in Human Phagocytes (THP-1 macrophages). *strain*, *8325*, p.4.

Pages, JM., James, CE., Winterhalter, M. 2008. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nature reviews Microbiology. 6,893-903.

Patejko, M., Jacyna, J., Markuszewski, M.J., 2017. Sample preparation procedures utilized in microbial metabolomics: An overview. Journal of Chromatography B. 1043, 150–157.

Paterson, GK., Harrison, E.M., Holmes, MA. 2014. The emergence of *mecC* methicillin-resistant *Staphylococcus aureus.* Trends in Microbiology. 22(1), 42-47.

Perreten, V., Giampa, N., Schuler-Schmid, U., Teuber, M. 1998. Antibiotic Resistance Genes in Coagulase-negative Staphylococci Isolated from Food Systematic and Applied Microbiology. 21, 113-120.

Philippon, A., Labia, R., Jacoby, G. 1989. Extended-spectrum beta-lactamases. Antimicrobial agents and chemotherapy. 33, 1131-6.

Pietrocola, M., Rodrigues, E., Bercot, F. *2021* Risk Society and Science Education. *Science and Education* 30, 209–233

Planchon, S., Gaillard-Martine, B., Dordet-Frisoni, E., Bellon-Fontaine, MN., Leroy, S., Labadie, J., Hebraud, M., Talon, R. 2006. Formation of biofilms by *Staphylococcus xylosus*. International Journal of Food Microbiology. 109, 88-96.

Riethmiller, S. 2005. From Atoxyl to Salvarsan: Searching for the magic bullet. Chemotherapy. 51, 234-242.

Roberts, MC. 2005. Update on acquired tetracycline resistance genes. FEMS microbiology letters. 245, 195-203.

Robicsek, A., Jacoby, GA., Hooper, DC. 2006. The worldwide emergence of plasmid-mediated quinolone resistance. The Lancet infectious diseases. 6, 629-40.

Rogers, GB., Carroll, MP., Bruce, KD. 2012. Enhancing the utility of existing antibiotics by targeting bacterial behaviour. British Journal of Pharmacology. 165, 845-857.

Rosenbach, F.J. 1884. Mikro-Organismen bei den. Wund-Infections-Krankheiten des Menschen. J.F. Bergman, editor. Wiesbaden, Germany. 1–122.

Rosenstein, R., Götz, F. 2013. What Distinguishes Highly Pathogenic Staphylococci from Medium- and Non-pathogenic? Current Topics in Microbiology and Immunology. 358,33-89 67

Rossolini, GM., Arena, F., Pecile, P., Pollini, S. 2014. Update on the antibiotic resistance crisis. Current Opinion in Pharmacology, 18:56–60

Rubin, RJ. et al. 1999. The economic impact of Staphylococcus aureus infection in New York City hospitals, Emergent Infectious diseases. 5, 9-17.

Seel, W., Baust, D., Sons, D., Albers, M., Etzbach, L., Fuss, J. and Lipski, A., 2020. Carotenoids are used as regulators for membrane fluidity by *Staphylococcus xylosus*. Scientific Reports, 10(1), pp.1-12.

Selden, R., Lee, S., Wang, WLL., Bennett, JV., Eickoff, TC. 1971. Nosocomial Klebsiella infections: intestinal colonization as a reservoir. *Annals of Internal Medicine*, *74*(5), 657-664.

Simmler, C., Napolitano, JG., McAlpine, JB., Chen,. Pauli, SG. 2014. Universal quantitative NMR analysis of complex natural samples, Current Opinion in Biotechnology, (52)51-59

Singh, V. 2013, Antimicrobial Resistance. Microbial pathogens and strategies for combating them: science, technology and education, Formarex 291-296.

Siqueira, J F., Lima, KC. 2002. *Staphylococcus epidermidis* and *Staphylococcus xylosus* in a secondary root canal infection with persistent symptoms: a case report. Australian Endodontic Journal. 28, 61–63.

Smith, PA., Romesberg, FE. 2007. Combating bacteria and drug resistance mechanisms of persistence and adaptation. Nature Chemical Biology. 3(9), 549-556

Spellberg, B. and Gilbert, D.N., 2014. The future of antibiotics and resistance: a tribute to a career of leadership by John Bartlett. Clinical infectious diseases, 59(suppl_2), S71-S75.

Tajkarimi, M., Ibrahim, S., Cliver., D. 2010. Antimicrobial herb and spice compounds in food. Food Control. 21, 1199.

Tiwari, BK., Valdramidis, VP., O'Donnell, CP., Muthukumarappan, K., Bourke, P., Cullen P. 2009. Application of natural antimicrobials for food preservation. Journal of Agricultural and Food Chemistry. 57, 5987.

Torjesen I. 2013 Antimicrobial resistance presents an "apocalyptic" threat similar to that of climate change, CMO warns. British Medical Journal, 346:f1597

Villa, PV. 2012, Enzybiotics and phages: Safe alternatives to antibiotics in the control of food. Microbiology Today. 39(4), 212-215.

Volkers, G., Palm, GJ., Weiss, MS., Wright, GD., Hinrichs, W. 2011. Structural basis for a new tetracycline resistance mechanism relying on the TetX monooxygenase. FEBS Letters. 585, 1061–1066.

Waksman, SA., Woodruff, HB. 1942. Streptothricin, a new selective bacteriostatic and bactericidal agent, particularly active against Gram-negative bacteria. *Proceedings of the Society for Experimental Biology and Medicine*, *49*(2), pp.207-210.

Waksman, SA., 1973. History of the word 'antibiotic'. *Journal of the history of medicine and allied sciences*, *28*(3), 284-286.

Wang, X., Wood, TK. 2011. Toxin-Antitoxin Systems Influence Biofilm and Persister Cell Formation and the General Stress Response, Applied and Environmental Microbiology, 76(10), 5577–5583

Webber, MA., Piddock, LJ. 2003. The importance of efflux pumps in bacterial antibiotic resistance. The Journal of Antimicrobial Chemotherapy. 51, 9-11.

WHO. 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery and, development of new antibiotics.

Wieland, B., Feil, C., Gloria-Maercker, E., Thumm, EG., Lechner, M., Bravo, JM., K. Poralla, K., Götz, F. 1994. Genetic and biochemical analyses of the biosynthesis of the yellow carotenoid 4,4-diaponeurosporene of Staphylococcus aureus. Journal of Bacteriology. 176. 7719–7726.

Wright, GD. 2005. Bacterial resistance to antibiotics: enzymatic degradation and modification. Advanced drug delivery reviews. 57, 1451-70.

Wu, S., De Lencastre, H., Tomasz, A. 1996. Sigma-B, a putative operon encoding alternate sigma factor of Staphylococcus aureus RNA polymerase: molecular cloning and DNA sequencing. Journal of Bacteriology. 178, 6036–6042.

Wu, X., Yu, H., Ba, Z., Chen. J., Sun, H., Han, B., 2010. Sampling methods for NMR-based metabolomics of *Staphylococcus aureus.* Biotechnology Journal. 5, 75-84.

Xia, J., Gao, J. and Tang, W., 2016. Nosocomial infection and its molecular mechanisms of antibiotic resistance. Bioscience trends, 10(1), pp.14-21.

Yonyama, H., Katsumata, R. 2006. Antibiotic resistance in bacteria and its future for novelle antibiotic development. Bioscience, Biotechnology and Biochemistry, 70(5), 1060-1075