EVALUATION OF CELL-FREE SUPERNATANT OF LACTIC ACID BACTERIA, LACTIPLANTIBACILLUS PLANTARUM K014 AGAINST SKIN PATHOGEN BACTERIA

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by

LARRY KURAI MLAMBO

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LIST OF SYMBOLS

x

α	Alpha
β	Beta
°C	Degree Celsius
±	Plus Minus
%	Percentage
g	Grams
h	Hours
L	Liter
min	Minute
mm	Millimeter
nm	Nanometer
μl	Microliter
μm	Micrometer
v/v	Volume Per Volume
w/v	Weight Per Volume
pH	Potential Of Hydrogen

LIST OF ABBREVIATIONS

ABTS   2,2'-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid)

ABS   Absorbance

ANOVA   Analysis Of Variance

ACME   Arginine Catabolic Mobile Element

BLIS   Bacteriocin-Like Inhibitory Substance

BSA   Bovine Serum Albumin

BMD   Broth Microdilution

CFS   Cell Free Supernatant

CFU   Colony Forming Unit

CTAB   Cetyltrimethylammonium Bromide

DDHP   2,2-Diphenyl-1-Picrylhydrazyl

DMSO   Dimethyl Sulfoxide

EMP   Embden-Meyerhof-Parnas pathway

FDA   Food And Drug Administration

FIC   Fractional Inhibitory Concentration

GC-MS   Gas Chromatography – Mass Spectrophotometry

GRAS   Generally Recognized As Safe

HA   Hyaluronic Acid

HMDS   Hexamethyldisilazane

HCl   Hydrochloric Acid

HPODE   Hydroperxyoctadeca-9,11-Dienoate

LAB   Lactic Acid Bacteria

LOX   Lipoxygenase

MIC   Minimum Inhibitory Concentration

MLST	Multilocus Sequence Typing
MLVA	Multiple-Locus Variable Number Of Tandem Repeats Analysis
MRS	Man Rogosa Sharpe
MRSA	Methicillin-resistant *Staphylococcus Aureus*
MSSA	Methicillin-susceptible *Staphylococcus Aureus*
NaCl	Sodium Chloride
NDGA	Nordihydroguaiaretic Acid
NADH	Nicotinamide Adenine Dinucleotide + Hydrogen
OD	Optical Density
PGFE	Pulsed Field Gel Electrophoresis
PVL	Panton-Valentine Leucocidin
ROS	Reactive Oxygen Species
RPM	Rotation Per Minute
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
rRNA	Ribosomal Ribonucleic Acid
SDF	Soluble Dietary Fiber
SDS page	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscope
UV	Ultravioloet

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KAJIAN SUPERNATAN BEBAS SEL DARIPADA BAKTERIA ASID

LAKTIK, LACTIPLANTIBACILLUS PLANTARUM K014 TERHADAP

BAKTERIA PATOGEN KULIT

ABSTRAK

Kajian ini bertujuan untuk menilai kesan metabolit bioaktif yang dihasilkan oleh bakteria asid laktik terhadap *Staphylococcus aureus* (MRSA) yang tahan methicillin ATCC43300. Sebanyak enam LAB telah dipilih untuk menilai aktiviti antimikrob terhadap MRSA ATCC43300, patogen kulit yang sangat tahan terhadap kebanyakan antibiotik. Pengasingan K014 daripada sayuran yang ditapai mencatatkan perencatan tertinggi terhadap MRSA ATCC43300 pada 91.93±0.36%. Penjujukan rRNA 16S mendedahkan bahawa pengasingan K014 berkait rapat dengan Lactiplantibacillus plantarum (dahulunya Lactobacillus plantarum) dan jujukan itu kemudiannya disimpan dalam pangkalan data Genbank dengan nombor aksesi MW180960, dinamakan sebagai *Lactiplantibacillus plantarum* K014. Dalam kajian kesan pH dan suhu terhadap aktiviti antimikrob CFS *L. plantarum* K014, CFS mempunyai toleransi yang tinggi terhadap perubahan suhu yang mengekalkan aktiviti antimikrob pada 121ºC selama 15 minit dan pH 6 berasid dengan perencatan peratusan 64.25±0.13 % dan 26.45±13.26%. Aktiviti perencatan CFS pada MRSA telah menurun sedikit daripada 100% kepada 84.62% dan 88.13% apabila ia dirawat dengan α-amilase dan proteinase K. Metabolit bioaktif seperti asid laktik dan asid hyaluronik dihasilkan oleh *L. plantarum* K014 dengan kepekatan asid laktik dalam CFS pada 9.58 ± 0.74 mg/mL manakala kepekatan asid hyaluronik pada 0.33 ± 0.01 mg/mL. Apabila diuji menggunakan ujian DDHP, CFS menunjukkan keupayaan antioksidan yang

lemah pada 2.97±0.57% berbanding nilai purata antioksidan ujian ABTS pada 46.28±5.33%. CFS menunjukkan aktiviti anti-radang yang tinggi untuk ujian lipoxygenase (LOX) pada 43.66±1.74% berbanding ujian hyaluronidase pada 3.66±1.66%. Imej mikroskopik menunjukkan lisis dinding sel bakteria dengan pembebasan kandungan sitoplasma berbeza dengan kawalan negatif dengan dinding sel yang utuh. Dalam kajian tersebut, CFS *L. plantarum* K014 telah dicirikan dengan menggunakan kromatografi Cecair Berprestasi Tinggi Fasa Songsang (RP-HPLC) dan Kromatografi Gas – Spektrometri Jisim (GC-MS). Kandungan peptida tertinggi yang diperhatikan dalam kromatografi Cecair Prestasi Tinggi Fasa Terbalik (HPLC) pada 280nm adalah untuk pecahan, puncak 2 (11.547) dan 4 (14.174) pada (50% A, 50% B) kepekatan fasa bergerak. Luas tertinggi liputan dan ketinggian puncak dalam Kromatografi Gas – Spektrometri Jisim (GC-MS) diperoleh pada 3.226 minit daripada asid lemak, gliserin dengan peratusan masing-masing 56.17% dan 35.47%. Kepekatan perencatan minimum (MIC) untuk CFS dalam analisis sinergi ialah 5.68 mg/ml. Sinergi lemah pada diperhatikan pada kepekatan CFS yang lebih tinggi (11.36 mg/ml) dan kepekatan amoksisilin yang lebih rendah (3.52μg/ml) dengan indeks FIC 0.92. Sebaliknya, sinergi kepekatan CFS yang lebih rendah (2.84 mg/ml) dan kepekatan Amoxicillin yang lebih tinggi (225 μg/ml) mempunyai indeks FIC sebanyak 0.96. Metabolit bioaktif *L. plantarum* K014 menunjukkan potensi yang sangat menjanjikan untuk digunakan pada patogen kulit topikal.

EVALUATION OF CELL-FREE SUPERNATANT OF LACTIC ACID BACTERIA, LACTIPLANTIBACILLUS PLANTARUM K014 AGAINST SKIN PATHOGEN BACTERIA

ABSTRACT

This study aims to evaluate the effects of bioactive metabolites produced by lactic acid bacteria against methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC43300. A total of six LAB were selected to evaluate the antimicrobial activity against MRSA ATCC43300, a skin pathogen that is highly resistant to most antibiotics. The K014 isolate from a fermented vegetable recorded the highest inhibition against MRSA ATCC43300 at 91.93±0.36%. 16S rRNA sequencing revealed that the K014 isolate was closely related to *Lactiplantibacillus plantarum* (*formerly Lactobacillus plantarum*) and the sequence was subsequently deposited in the Genbank database with an accession number of MW180960, named as *Lactiplantibacillus plantarum* K014. In the study of effects of pH and temperature on the antimicrobial activity of the CFS of *L. plantarum* K014, the CFS had high tolerance to temperature changes retaining the antimicrobial activity at 121°C for 15 minutes and acidic pH 6 with percentage inhibition of 64.25±0.13% and 26.45±13.26% respectively. The inhibition activity of CFS on MRSA was slightly decreased from 100% to 84.62% and 88.13% when it was treated with α-amylase and proteinase K, respectively. The bioactive metabolites such as lactic acid and hyaluronic acid were produced by *L. plantarum* K014 with lactic acid concentration in CFS at 9.58 ± 0.74 mg/mL while the hyaluronic acid concentration at 0.33 ± 0.01 mg/mL. When tested using the DDHP assay, the CFS demonstrated weak antioxidant capabilities at

2.97±0.57 % compared to the ABTS assay's average antioxidant values at 46.28±5.33 %. The CFS showed high anti-inflammatory activity for lipoxygenase (LOX) assay at 43.66±1.74% relative to the hyaluronidase assay at 3.66±1.66%. Microscopic images showed the lysis of the bacterial cell walls with the release of cytoplasmic contents in contrast to the negative control with the intact cell walls. In the study, the CFS of *L. plantarum* K014 has been characterized using the Reverse Phase High Performance Liquid chromatography (RP-HPLC) and Gas Chromatography – Mass Spectrometry (GC-MS). The highest peptides content observed in Reverse Phase High Performance Liquid chromatography (RP-HPLC) at 280nm were for fractions, peak 2 (11.547) and 4 (14.174) at (50% A, 50% B) concentration of the mobile phase.The highest area coverage and height of peak in Gas Chromatography – Mass Spectrometry (GC-MS) was obtained at 3.226 minutes from the fatty acid, glycerine with a percentage of 56.17% and 35.47% respectively. The minimum inhibitory concentration (MIC) for the CFS in the synergy analysis was 5.68 mg/ml. Weak synergy was observed at higher concentration of CFS (11.36 mg/ml) and lower concentration of amoxicillin (3.52μg/ml) with a FIC index of 0.92. In contrast, synergy at a lower concentration of CFS (2.84 mg/ml) and higher concentration of Amoxicillin (225 μg/ml) had a FIC index of 0.96. The bioactive metabolites of *L. plantarum* K014 showed very promising potential to be used on topical skin pathogens.

CHAPTER 1

INTRODUCTION

1.1   Overview

Skin problems have significantly impacted both primary and secondary infections in people all over the world over the past century. Most primary skin pathogens, such as *Staphylococcus aureus*, group A-hemolytic streptococci, and coryneform bacteria, can result in the development of these illnesses in the largest organ of the body (Swetha and Pandian, 2019). Skin infections, such as impetigo, impetigo contagiosa, ecthyma, folliculitis, "impetigo of Bockhart," furuncle, carbuncle, tropical ulcer, etc., are referred to as pyoderma. *S. aureus* is well established to contribute significantly to the pyoderma development, and the evidence suggests that beta haemolytic streptococci (BHS), particularly group A, continue to be the primary or secondary cause of the condition in many tropical regions, despite reports to the contrary in more recent studies (World Health Organization, 2005). 2044 (14.9%) of the 30221 *Staphylococcus aureus* isolates from clinical samples in 2020 were confirmed to be MRSA, according to the Ministry of Health (MOH) in the Malaysia National Antibiotic Resistance Surveillance Report 2020. The majority of these isolates came from blood (22.4%), pus (18%), and tissue (16.9%), with 2044 (14.9%) coming from tissue samples. Most MRSA isolates came from patients in the surgical, orthopedic, and medical wards (Ministry of Health Malaysia, 2020).

Severe sepsis, pneumonia, toxic shock syndrome, and endocarditis are just a few of the severe and life-threatening diseases that *Staphylococcus aureus* has been linked to in opportunistic human infections (Otto, 2010). *S. aureus* can produce a variety of virulence factors, such as peptidoglycan-covalently-attached surface proteins and are also known as cell wall-anchored proteins (CWA). An average of 24 distinct CWA proteins are present in *S. aureus*, which helps it avoid detection by the host immune system (Foster et al., 2014).

To find new bioactive substances, scientists are actively using microorganisms in their research. Bacteria typically have an edge in this regard since humans can use and explore the advantages over an extended period because they are much easier to culture. Lactic acid bacteria are one of the prospective microorganisms that can be studied and offer considerable chances for the discovery of novel bioactive compounds (Barcenilla et al., 2022). A group of Gram-positive, anaerobic bacteria known as lactic acid bacteria (LAB) produce lactic acid into the medium as the primary fermentation product along with various secondary metabolites. Through the suppression of opportunistic *S. aureus*, the bioactive metabolites from *Lactobacillus* sp. have been shown to have wound healing effects (Ong et al., 2020). According to recent research (Ishikawa et al., 2020; Sürmeli et al., 2019), investigations on the CFS or bacteriocins produced by lactic acid bacteria to prevent the growth of MRSA have attracted a lot of attention. In one study, the glycolipodepsipeptide ramoplanin and the most thoroughly researched lantibiotic, nisin, which affects the lipid II precursor molecule, were combined. Of the 20 MRSA strains assessed, the combination resulted in 14 strains exhibiting synergistic interactions (Brumfitt et al., 2002).

1.2   Problem statement

Although *S. aureus* is susceptible to the majority of antibiotics ever created, antibiotic resistance frequently develops as a result of horizontal gene transfer from outside sources. Hospital- and community-acquired strains of the gram-positive, facultative anaerobic pathogen *S. aureus* exist. Even though *S. aureus* has historically been opportunistic, several strains are now extremely pathogenic. It is the most prevalent skin bacteria, with 20% of people persistently harboring at least one strain and 60% of people carrying it intermittently (Morehead and Scarbrough, 2018). Community infections in certain epidemics have been linked to MRSA. Methicillin resistance imparts resistance to all penicillinase-resistant penicillins and cephalosporins because of the mec gene's highly evolving nature, which is located on the Staphylococcal chromosomal cassette mec (SCCmec) region of the bacterial chromosome (Liu et al., 2016). Additionally, *S. aureus* has evolved to become resistant to several widely used antibiotics, including penicillin, oxacillin, vancomycin, and daptomycin. Treatment and therapeutic options have been further limited by the advent of antibiotic-resistant bacteria. Natural alternatives are therefore required to treat *S. aureus*, either as stand-alone treatments or in conjunction with synthetic medications (Rodvold and McConeghy, 2014). The conventional approach to addressing the issue of antibiotic resistance would be to create new antibiotics that are more effective against the resistant bacteria. The method has resulted in the development of various MRSA-treating medications during the past ten years, including linezolid, daptomycin, tigecycline, and televancin (Rodvold and McConeghy, 2014).

Nevertheless, despite the achievement, there is much skepticism regarding the likelihood of a new antibiotic being able to combat multidrug-resistant bacteria. Major pharmaceutical corporations' interest has also been dampened by regulatory barriers. Recently, numerous pharmacological groups, including antibiotics, have seen a drop in the tolerance of unpleasant side effects. Clinical trial approval requirements have frequently increased from demonstrating non-inferiority to superiority, and occasionally the lack of clear study protocols, particularly for antibiotics, has hampered the development. Pharmaceutical firms are faced with a dilemma where federal agencies urge the development of antibiotics while concurrently enacting regulations limiting the use of that very development (Fair and Tor, 2014).

1.3   Objectives

Main Objective: Production of CFS by *Lactiplantibacillus plantarum* K014 against MRSA ATCC43300 and evaluation of its antibacterial, antioxidant and anti-inflammatory activities

To accomplish the study's principal goal, three objectives were carried out:

1. To investigate the antimicrobial properties of CFS produced by *Lactiplantibacillus plantarum* K014 against MRSA ATCC43300.

2. To characterize and evaluate the potential of CFS produced by *Lactiplantibacillus plantarum* K014 as antioxidant and anti-inflammatory agents.

3. To determine the synergistic effect of CFS produced by *Lactiplantibacillus plantarum* K014 with antibiotics against MRSA ATCC43300.

CHAPTER 2

LITERATURE REVIEW

2.1   Skin infections

Around 300 million people globally contract various kinds of skin infections each year, which has an impact on the human population overall. Fungal skin illnesses and acne vulgaris were ranked among the top 10 most common skin diseases in a 2010 survey by the Global Burden of Disease (GBD). In addition, the study found that skin disorders were the 4th most common non-fatal illness burden and the 2nd to 11th main cause of years lived with disability (Balakrishnan et al., 2016). More than 1,000 diseases of the skin or connected to the skin are listed in the International Classification of Diseases, with a few categories controlling the majority of the burden of skin diseases as shown in Figure 2.1 (Hay et al., 2014). While the prevalence rate of skin infections was observed to range from 20% to 80% in developing nations. This is corroborated by research conducted in underdeveloped nations like Mauritius, where adults had a prevalence rate of skin infections of 33%, compared to 42% among African populations in Sierra Leone (Balakrishnan et al., 2016). As a consequence of violence, persecution, and conflict, there are 70.8 million displaced people worldwide. Due to several interconnected conditions, including poor housing, exposure to the elements, inadequate nutrition, congestion, violence, and problems in healthcare infrastructure, cutaneous disorders are frequently observed among displaced people (Knapp et al., 2020).

Rapid diagnostic tests (such as those for malaria, TB, and HIV), hemograms, and fundamental chemistries are generally the only diagnostic options available in refugee camps. The ability to process histologic samples or do tissue cultures is typically limited to nonexistent, while certain facilities might have access to Gram stains and acid-fast bacilli smears (Knapp et al., 2020). In both developing and wealthy nations, a significant role in the emergence of skin diseases is a lack of knowledge of risk factors (Goonmatee and Rajesh, 2013). According to studies, most people are aware of the possible adverse effects of medications used to treat skin infections. However, there is a lack of information about skin infection risk factors, which makes it difficult for physicians or pharmacists to recognize symptoms right away. Where awareness is a concern, this level of understanding may be especially relevant (Ramamuthie et al., 2015). In Malaysia, customers purchase medicines based on their personal experience or knowledge of how well they work for their particular skin issue. The most frequent skin infections in older people, which result in repeated visits and hospital admissions, are eczema, xerosis, and dermatitis. Age, alcohol use, pharmacological treatments, and the prevalence of co-morbidities in the older population are some factors that determine how well skin infections are treated (Akhtar et al., 2021). Although skin conditions are common, treatment priority is lower since less is known or understood about the more serious downstream consequences among displaced people. Large quantities of bulky, heavy topical medications used to treat dermatologic disorders are expensive to ship, especially to remote locations with dangerous driving conditions. Additionally, in low- and middle-income nations hosting refugees, the local accessibility of dermatological drugs may be restricted (Knapp et al., 2020).
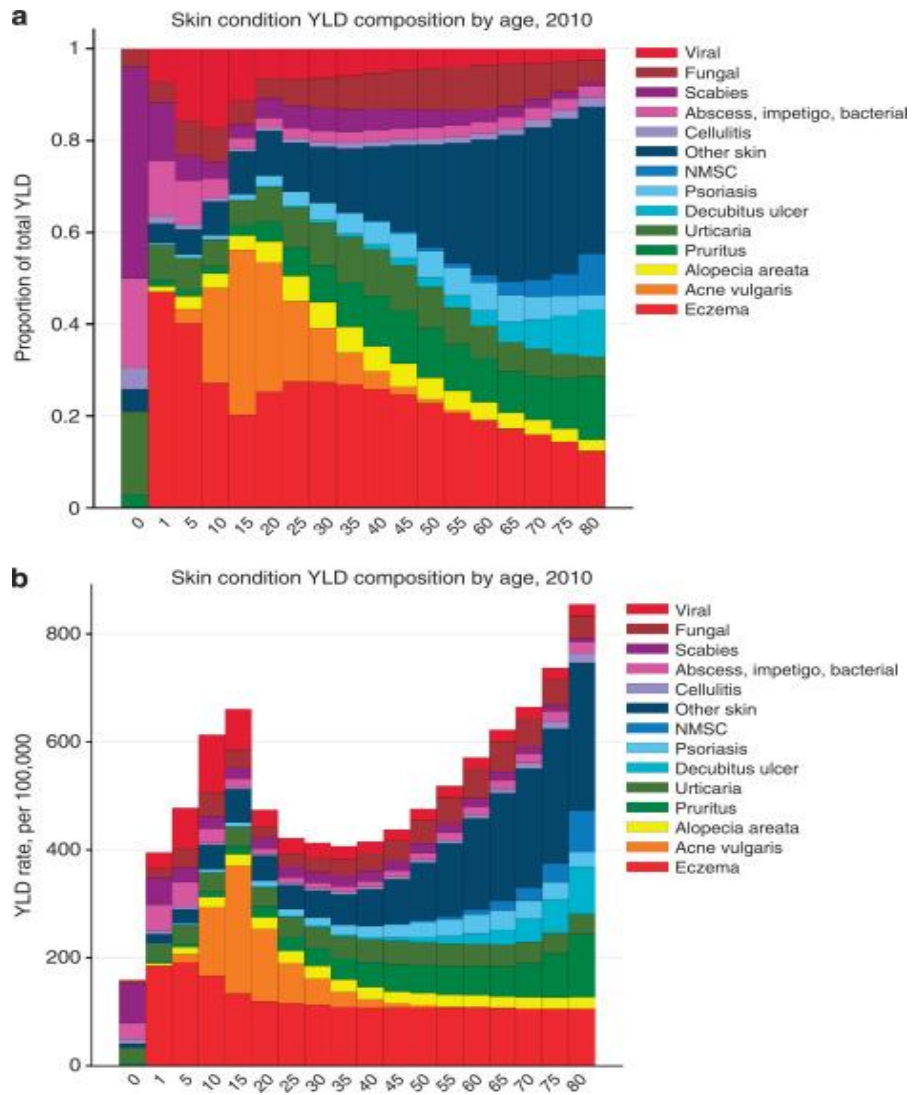
Figure 2.1 Skin condition years lost due to disability (YLD) composition by age, 2010. [1] (Source: Hay et al., 2014).

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[1] (a) Proportion of total YLD. (b) YLD rate per 100,000. NMSC, non-melanoma skin cancer

2.2 Emergence of antimicrobial resistance

Millions of people's lives have been impacted by the global uptick in incidences of bacteria that are multi-drug resistant (Avner et al., 2012; Morehead and Scarbrough, 2018). Antibiotic resistance is a complicated, multifaceted problem. Resistance was discovered shortly after antibiotics were discovered (Table 2.1). *Staphylococcus* penicillin resistance was already known before the 1943 introduction of the first antibiotic and the widespread manufacture of penicillin. According to research, this sort of resistance could not have developed in the time since the discovery of penicillin due to the lack of genetic variety required. This finding suggests that bacteria probably possess a long-evolving innate preference for resistance that is preserved within their DNA (Fair and Tor, 2014; McDermott et al., 2002; Ventola, 2015). Globally, the pandemic has sparked concern by demonstrating how it would impair future generations' ability to treat infections in both developed and underdeveloped nations. Prospective models have also shown how uncertain antibiotic medicines will be in the twenty-first century (Capita and Alonso-Calleja, 2011)

2.3   Antibiotics history

Table 2.1 Timeline of antibiotic development, resistance, and global response.[2]

1928	Alexander Fleming discovers penicillin
1930s	Prontosil, a sulfonamide, is first commercially available antibacterial
1940	Penicillin-R *Staphylococcus* identified
1943	Florey and Chain efficiently purify and scale-up production of penicillin
1940–1962	The golden age of antibiotic discovery and production
1950	Tetracycline introduced
1953	Erythromycin introduced
1955	Penicillin use is restricted to prescription only due to widespread misuse
1959	Tetracycline-R *Shigella* identified
1960	Methicillin introduced
1962	Methicillin-R *Staphylococcus* identified
1962	Nalidixic acid introduced (predecessor to fluoroquinolones)
1965	Penicillin-R pneumococcus identified
1967	Gentamicin introduced
1968	Erythromycin-R *Streptococcus* identified

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[2] The dates are based on the first written accounts of resistance. The date for PDR *Acinetobacter* and *Pseudomonas* is determined by reports of outbreaks or health care transmission. PDR stands for "pan drug-resistant," R stands for "resistant," WHO stands for "World Health Organization," and XDR stands for "extensively drug-resistant." Adapted from Centers for Disease Control and Prevention. Antibiotic resistance threats in the United States. 2013. Available at: https://www.cdc.gov/drugresistance

Table 2.1 Timeline of antibiotic development, resistance, and global response (Continued)

1972	Vancomycin introduced
1979	Gentamicin-R *Enterococcus* identified
1985	Imipenem and ceftazidime introduced
1987	Ceftazidime-R Enterobacteriaceae identified
1988	Vancomycin-R *Enterococcus* identified
1996	Levofloxacin introduced
1998	Imipenem-R Enterobacteriaceae identified
2000	Linezolid introduced
2000	XDR tuberculosis identified
2001	Linezolid-R *Staphylococcus* identified
2001	WHO launches Global Strategy for Containment of Antimicrobial Resistance
2002	Vancomycin-R *Staphylococcus* identified
2003	Daptomycin introduced
2004/5	PDR-*Acinetobacter* and *Pseudomonas* identified
2008	NDM-1, the New Delhi metallo-carbapenemase, observed in Enterobacteriaceae
2009	Ceftriaxone-R *Neisseria gonorrhoeae* and PDR-Enterobacteriaceae identified
2010	Ceftaroline introduced
2011	Ceftaroline-R *Staphylococcus* identified
2013	CDC Report on Antibiotic Resistance Threats in the United States
2014	WHO releases Antimicrobial Resistance: Global Report on Surveillance
2015	WHO releases Antibiotic Resistance: Multi-Country Public Awareness Survey
2015	WHO launches Global Action Plan on Antimicrobial Resistance

Penicillin's use by soldiers surged during World War II, helping to keep bacterial infections under control throughout the 1940s. The emergence of a clinical issue involving bacteria's resistance to penicillin in the early 1950s prompted the creation of beta-lactam antibiotics. MRSA was first linked to transferable multidrug resistance in the early 1960s in both the U.K and U.S.A (McDermott et al., 2002; Ventola, 2015). It takes a comprehensive analysis to trace the evolutionary development of an antibiotic or an antibiotic family shown in Table 2.1. Finding the common ancestor between two members of the same antibiotic family presents another issue. Lack of consideration for both the origins of the genes that make antibiotics and the ecological significance of the antibiotic in the producer's natural niche clouds the identification of an early ancestor (Clardy et al., 2009; Fair and Tor, 2014). Genes involved in producing secondary metabolites, such as antibiotics, provide evidence of horizontal gene transfers in the bacterial genes' evolutionary ancestry. The identification of genes for regulation, biosynthesis, and resistance in clusters along the extensive DNA of diverse bacteria is also a result of horizontal gene transfer. As a result, it can be inferred that a microbe's repertoire of secondary metabolites is more dependent on its neighbors than on its predecessors (Clardy et al., 2009). By lowering mortality and morbidity, antibiotics have had a significant impact on society. After the development of the first antimicrobials, drug discovery secured the availability of several medications, giving clinicians a variety of alternatives to lessen the burden of previously life-threatening diseases. But the reliance on antibiotics without considering the consequences gave rise to a new era of pathogens that are resistant to medications, which has plagued many practitioners (Zaffiri et al., 2012).

2.4 Aspects causing the rise in antibiotic resistance

There is antibiotic resistance on every continent. Efficient antibiotics are essential for both preventing and treating infectious diseases, but the pharmaceutical industry is only developing a small number of brand-new, cutting-edge medicines. Finally, the global resistance conundrum is heavily influenced by concerns about access and overabundance. The decline in research and development of newer antibiotics is caused by a number of variables. Many of the largest pharmaceutical companies have reduced or eliminated their antibiotic sections due to insufficient profitability (Fair and Tor, 2014). Short-course and limited usage, increased compliance costs, generic competitiveness, and unavoidable resistance have all contributed to a poor risk-to-reward ratio, which will likely lead to future declines in medication consumption. In the end, only four international pharmaceutical corporations still have divisions dedicated to developing antibiotics. As a result, both US and international organizations have implemented laws that provide incentives for businesses to advance the development of antibiotics (Morehead and Scarbrough, 2018)

2.5 Methicillin-resistant *Staphylococcus aureus*

A common Gram-positive nosocomial pathogen, *S. aureus* is linked to numerous ailments, ranging from mild skin infections to more severe and perhaps fatal infections including toxic shock syndrome and bacterial endocarditis. The ability of these strains to rapidly grow and acquire antibiotic resistance has led to the emergence of multidrug-resistant bacteria like MRSA (Che Hamzah et al., 2019).

In its global report from 2014, the World Health Organization (WHO) listed MRSA as a major influence among the seven illnesses of global concern. MRSA has been connected to higher cases of septic shock and fatalities than methicillin-susceptible *Staphylococcus aureus* (MSSA). The prevalence of MRSA among clinical isolates of *S. aureus* in Malaysia ranged from 14.2% to 23.1%, with rates of 15% and 14.9% for the years 2019 and 2020, respectively as shown in Figure 2.2 (Ministry of Health Malaysia, 2020). MRSA has infiltrated clinics worldwide with rising rates of infection and isolation since its initial detection in 1961. MRSA was projected to cause 100 000 healthcare-related infections in the United States alone each year, and around 19 000 fatalities were linked to MRSA infection, exceeding the combined mortality of patients with HIV infection and AIDS (Li et al., 2015). MRSA was initially identified in hospital-acquired infections (HA-MRSA), but since then, it has extended to the general populace and is now referred to as community-acquired MRSA (CA-MRSA).

Functional genomic features serve as a general means to delineate the HA-MRSA and CA-MRSA. Previous studies have shown that HA-MRSA strains typically have relatively large SCCmec type I-III and are more recalcitrant to other classes of antibiotics, whereas CA-MRSA strains generate potent toxins and virulence factors like arginine catabolic mobile element (ACME), phenol-soluble modulins, and Panton-Valentine leucocidin (PVL) (Jones et al., 2021; Wu et al., 2019).
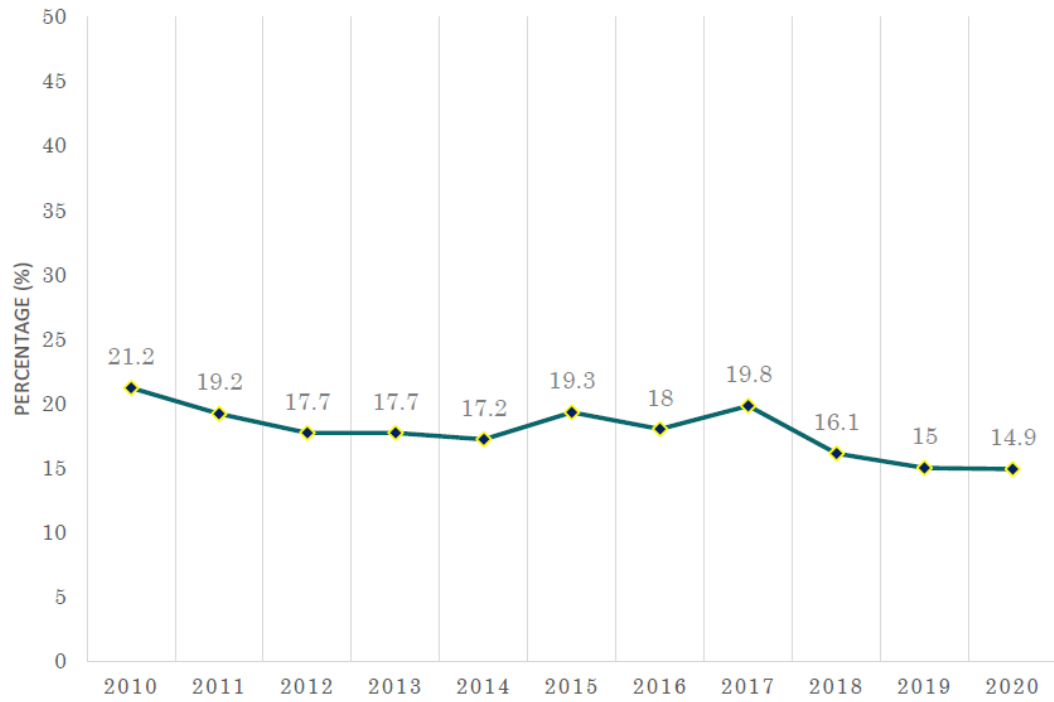
Figure 2.2 Trends of MRSA infection in Malaysia between 2010 and 2020.

Source: https://www.imr.gov.my/images/uploads/NSAR/2020/NSAR-REPORT 2020.pdf .

2.6   Genetic characterization of MRSA

Multiplex PCR, multilocus sequence typing (MLST), SCCmec typing, Pulsed field gel electrophoresis (PGFE), spa typing and multiple-locus variable number of tandem repeats analysis (MLVA), are some of the molecular typing tools that can be used to define the *S. aureus* population structure (Enright et al., 2002; Milheiriço et al., 2007; Tenover et al., 2007). Spa typing is a reliable and discriminatory technique based on the sequencing analysis of variable-number tandem repeats in the putative region X of the spa gene that encodes for staphylococcal protein A (SpA) (Asadollahi et al., 2018). The X region consists of a variable number of 24-bp repeats surrounded by well-conserved segments as shown in Figure 2.3. Numerous technological benefits, including speed, reproducibility, and portability, are combined in this single-locus sequence-based typing technique. Additionally, the spa locus' repeat nature allows it to index both micro-and macro variations at the same time, making it possible to utilize spa typing in both regional and worldwide epidemiological research (Hallin et al., 2009). There are various trends in the spa-type dispersion in isolates of *S. aureus* in various geographical regions of the world. Sustained epidemics are frequently caused by the ongoing emergence of novel strains that are constantly changing over time. According to several studies, some *S. aureus* lineages have a noticeable tendency toward hematogenous complications (Jones et al., 2021).
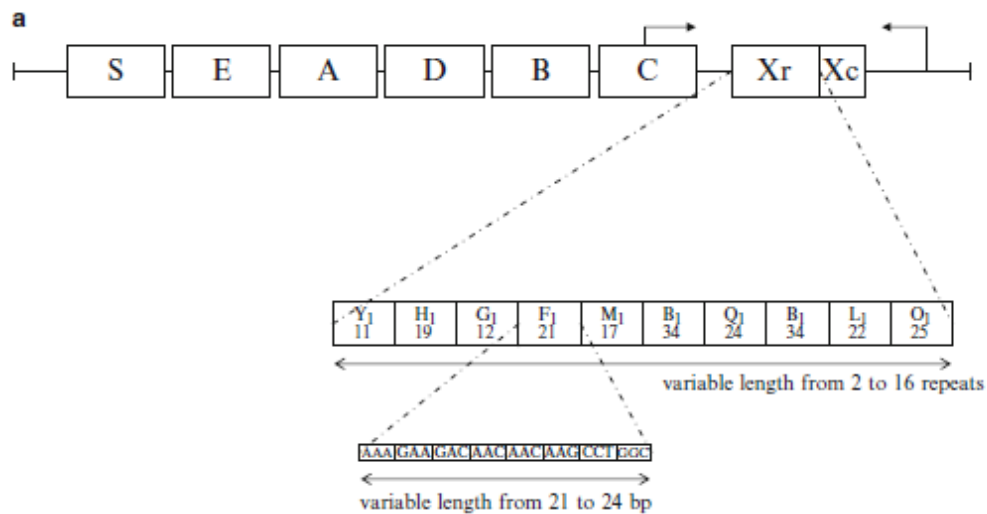
Figure 2.3 Schematic map of the spa gene.[3] (Source: Hallin et al., 2009).

Among these techniques, PFGE utilizing SmaI, which has been used by many researchers to investigate the epidemiology of MRSA and is regarded as the gold standard for genotyping MRSA. PFGE has already been used to type MRSA in Malaysia (Norazah et al., 2003, 2001). However, PFGE is a labor- and technically-intensive procedure. Additionally, it offers an opportunity for subjectivity in its interpretation, making it difficult to compare inter-laboratory data and requiring rigorous adherence to defined methods and interpretation criteria (Cookson et al., 1996; Murchan et al., 2003).

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[3] S is a signal sequence; A to D are IgG-binding domains; X is the C-terminal part, divided in two regions, the VNTR region (Xr) and a constant region coding for cell wall attachment (Xc). Arrows indicate the primers' localization.

The PCR-based, MLVA analysis also takes advantage of the intrinsic variability found in many repetitive DNA sequences. The main disadvantage is that epidemiological concordance may be compromised by repeated DNA evolution that is too rapid (Arricau-Bouvery et al., 2006). It is possible to determine whether two isolates are identical only by chance when the mutation frequency at a locus and the prevalence of certain alleles in a population are known. Other fragment-based techniques cannot achieve this. Additionally, as with all techniques that estimate molecular size using standard curves, it is difficult to size fragments accurately, even when utilizing fluorescent detection devices, because mobility depends both on the length and composition of the sequence. (Van Belkum et al., 2007).

Multilocus sequence typing (MLST), which relies on the sequence heterogeneity of around 500-bp length portions of seven housekeeping constitutive genes, was created to evaluate the genetic make-up of the *S. aureus* population. Application of this method to large *S. aureus* strain collections revealed that *S. aureus* population structure is fundamentally clonal and that the overwhelming portion of epidemic MRSA clones belong to a tiny proportion of phylogenetically different lineages or clonal complexes (CCs) (Enright et al., 2002). Additionally, MLST has shown to be sufficient for protracted global epidemiology and the investigation of current *S. aureus* transformation. However, MLST typing is still costly and time consuming to be used for routine surveillance and epidemic investigations (Hallin et al., 2009).

2.7   Mechanism of infection/ resistance

Based on the existence of a mec staphylococcal cassette chromosomal (SCCmec), it has been established that *S. aureus* evolved from MSSA. SCCmec also has the ccr gene complex, which produces recombinases necessary for SCCmec's mobility, in addition to the mec gene complex, which is made up of the mecA gene and its regulators, mecR1 and mecI. A number of SCCmec types have been discovered by coupling the class of the mec gene complex and the ccr allotype (Milheiriço et al., 2007). By combining these two genes, five primary subtypes have also been identified: SCCmec type I [Class B mec and type 1 ccr] , type II [Class A mec and type 2 ccr] , type III [Class A mec and type 3 ccr] , type IV [Class B mec and type 2 ccr], and type V [Class C mec and type 5 ccr] (Thong et al., 2009). The remaining parts of SCCmec are referred to as J regions (regions J1 - J3), which are quasi elements of the cassette even though they occasionally contain additional antibiotic resistance markers. The regions J1, J2, and J3 are located between the chromosomal left junction and the ccr complex, the mec complex, and the chromosomal right junction, respectively. Therefore, J1-ccr-J2-mec-J3 can be used to summarize the structural organization of SCCmec. SCCmec subtypes are defined by variations in the J regions within the same mec-ccr combination (Milheiriço et al., 2007). By horizontally transferring the low-affinity penicillin-binding protein 2a (PBP2a) gene from coagulase-negative staphylococcal species, the mecA gene, the bacteria can develop resistance to a variety of β-lactam antibiotics (Wu et al., 2019). However, a newly discovered gene that is now known as mecC revealed 69% sequence homology to the original mecA gene. Additionally, the mecC gene has demonstrated MRSA's resistance to cefoxitin and oxacillin (Lakhundi and Zhang, 2018).

Contrary to other SCCmec elements, SCCmec IV and SCCmec I do not encode additional resistance determinants; however, SCCmec IV does code for mecA .This protein may interact with the extracellular protein PVL since it is expressed on the cytoplasmic membrane's surface (Jones et al., 2021; Vandenesch et al., 2003). The prevalence of CA-MRSA infections appears to be increasing globally. Among the toxin genes generated by *S. aureus*, and MRSA infections associated with PVL include cutaneous sepsis and necrotizing pneumonia (Vandenesch et al., 2003). By binding to the immunoglobulin and rendering the bacteria inaccessible to opsonins, the SpA is a surface protein that contributes to the pathogenesis of *S. aureus* and prevents phagocytosis. The number of repeats in the spa region ranges from 21 to 27 bps (Asadollahi et al., 2018).

According to the availability of mobile genetic elements (MGEs) whose genes express various virulence factors and toxins, each strain of *S. aureus* has a variable level of virulence, as illustrated in Figure 2.4. While the genes for toxins, are present in all *S. aureus* strains, the genes for many secreted virulence factors, such as enterotoxins, exfoliative toxins A and B, and superantigen toxins (SaPIs), are found in accessory MGEs like transposable elements, pathogenicity islands (PIs), prophages, and plasmids (Kırmusaoğlu, 2017; Otto, 2010).
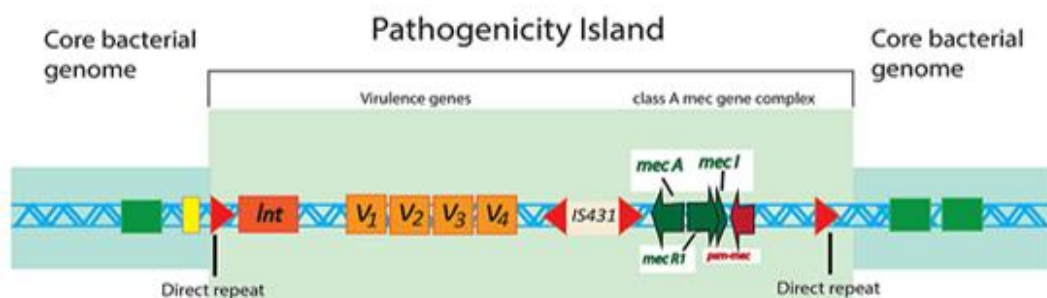

Figure 2.4 Staphylococcal genome. (Source: Kırmusaoğlu, 2017).

2.8   Pharmacotherapy to combat methicillin-resistant *Staphylococcus aureus*

Each hospital has a distinct pattern of resistance to antimicrobials, hence the antimicrobials that are effective against MRSA differ between hospitals. Therefore, understanding MRSA's clonal connection and its patterns of antimicrobial susceptibility can assist hospital infection control efforts in keeping an eye on and preventing the spread of MRSA within and between hospitals (Thong et al., 2009). The majority of benign Skin and Soft Tissue Infections (SSTIs) suspected of MRSA infection are treated empirically with antibiotics like clindamycin, tetracyclines, and trimethoprim/sulfamethoxazole orally (Khan et al., 2018).

When vancomycin is inaccessible or not tolerated, daptomycin is an appropriate parenteral substitute. Currently, daptomycin has received approval from both the FDA and EMEA for the treatment of complicated SSTIs. The use of daptomycin has been authorized by the FDA for the treatment of right-sided infective endocarditis and *S. aureus* bacteremia (French, 2006). If accessible and deemed cost-effective, newer medications including linezolid, tedizolid, and delafloxacin can also be utilized as alternatives to oral regimens. In addition to dalbavancin and oritavancin, which have a lengthy half-life, ceftaroline and telavancin are fast-acting alternatives. No matter which empiric antibiotic is initially chosen, ongoing treatment should be customized based on a thorough examination of culture and susceptibility data (Siddiqui and Koirala, 2022). Despite being frequently utilized off-label to treat SAB, ceftaroline is only supposed to be used to combat acute bacterial SSTIs and pneumococcal disease brought on by *S. aureus*. According to recent multicenter research, 70% of patients with MRSA bacteremia who received ceftaroline as a backup therapy, in combination with yet another antistaphylococcal drug or either alone, saw clinical success (Hassoun et al., 2017).

Staphyloxanthin is an antioxidant scavenger present in cell membranes and gives MRSA structural integrity, thymol treatment that inhibits this pigment lowers the polarization index, which has an impact on how long MRSA can survive in the presence of ROS. Previous research has demonstrated that decreasing staphyloxanthin pigment made MRSA cells more vulnerable to membrane-targeting drugs (Valliammai et al., 2021). Mupirocin 2% topical ointment is probably a good alternative for treating mild infections (like impetigo) and secondarily infected skin lesions (such as ulcers, lacerations, and eczema). A proper incision and drainage should still be the main therapeutic goal for cutaneous abscesses (I&D). According to recommendations, moist heat may be sufficient for smaller furuncles to encourage drainage (Khan et al., 2018).

2.9   Lactic acid bacteria

If given the right carbohydrate sources, lactic acid bacteria (LAB), a genus of Gram-positive, anaerobic, non-spore-forming bacteria, produce lactic acid as the main catabolism product into the medium (Kieliszek et al., 2021; Teuber, 1993). LAB typically are found in areas with abundant food sources. They can be found in cavities in both humans and animals as well as fermented foods, rotting fruit, plant materials, and dairy products (König and Fröhlich, 2017). They are a component of the gut's healthy microbiota because they are generally recognized as safe for humans and animals (GRAS) and have numerous positive benefits for human health without causing any substantial negative effects. Hence, lactic acid bacteria have become more and more important (König and Fröhlich, 2017). Fermented foods have played a significant role in the human diet since they helped preserve perishable raw materials and frequently added vitamins through microbial development. The manufacturing of fermented foods has grown significantly in the food processing sector since the turn of the century (Axelsson and Ahrné, 2000).

During research using heated milk to disprove PASTEUR'S germ theory, Joseph Lister (1873) accidentally discovered the first pure culture of bacteria. Bacterium lactis was the name he gave it. Although Lohnis (1909) designated this species as *Streptococcus lactis*, its current recognized name is *Lactococcus lactis* (Teuber, 1993). Orla-Jensen (1919) divided the LAB into seven genera based on end-product synthesis, morphology, and development at specific temperatures. However, because it makes use of features that are simply determinable in a typical bacteriological, Orla-Jensen's method is still highly beneficial for practical application (Axelsson and Ahrné, 2000).

The LAB group was composed of *Lactobacillus* (with Orla-Jensen's designations "Betabacterium," "Streptobacterium," and "Thermobacterium" included as subgenera), *Leuconostoc* ("Betacoccus"), *Pediococcus* ("Tetracoccus"), and *Streptococcus* after some taxonomic revisions, where essentially previously described genera were recognized as being identical. Until the development of molecular taxonomy in the 1970s and 1980s, these genera held a stable position in the systematics of lactic acid bacteria (Axelsson and Ahrné, 2000; Enany, 2018). The fact that lactic acid-producing bacteria come from a variety of genera, including *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*, reflects this biochemical definition. They may be as unlike phylogenetically as the *Actinomyces* branch (*Bifidobacterium*) and the *Clostridium* branch (all others) in the evolution of bacteria (Teuber, 1993).

The lactic acid bacteria (LAB) group, which has a rich history of usage in the safe production of fermented beverages and foods, plays a crucial role in these processes (Leroy and De Vuyst, 2004). These bacteria produce antimicrobial chemicals, which are crucial for ensuring food safety and extending the shelf life. Natural inhibitory substances may be used to replace or reduce the usage of chemical additives in food preservation as customer demand for "natural" and "additive-free" products grows (Müller et al., 2009; Pisoschi et al., 2018).