ANTIMICROBIAL ACTIVITIES OF ENDOPHYTIC FUNGI AND BACTERIA FROM *IPOMOEA AQUATICA*, *MANIHOT ESCULENTA*, AND *COLEUS AROMATICUS* ON EXTENDED-SPECTRUM BETA-LACTAMASE (ESBL)-PRODUCING *ESCHERICHIA COLI* AND METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

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(D18A0015)

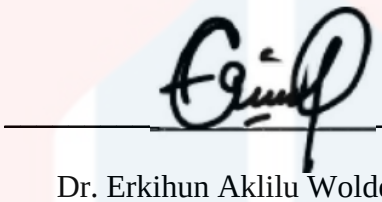
A RESEARCH PAPER SUBMITTED TO THE FACULTY OF VETERINARY MEDICINE, UNIVERSITI MALAYSIA KELANTAN
IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR
THE DEGREE OF
DOCTOR OF VETERINARY MEDICINE

FEBUARY 2023

UNIVERSITY MALAYSIA KELANTAN

CERTIFICATION

This is to certify that we have read this research paper entitled **'Antimicrobial Activity of Endophytic Fungi and Bacteria from *Ipomoea aquatica, Manihot esculenta,* and *Coleus aromaticus* on Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Escherichia coli* and Methicillin-resistant *Staphylococcus aureus* (MRSA)'** by Lim Chee Chee, and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT 55204 - Research Project.



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ACKNOWLEDGEMENT

Special thanks to those who have given their support for the completion of this project paper:

My supervisor Dr. Erkihun Aklilu Woldegiorgis

My co-supervisor Dr. Choong Siew Shean

All the laboratory assistance

My beloved parents and siblings

My partner Tan Yong Sheng

&

Faculty of Veterinary Medicine, University Malaysia Kelantan

Thank You

DEDICATION

I dedicate my dissertation work to all of the lecturers who helped me and motivated me to do better throughout the process. Special thanks to my supervisor, Dr. Erkihun Aklilu Woldegiorgis and co-supervisor, Dr. Choong Siew Shean for their guidance, support, encouragement, and active participation throughout the entire process. Without them, the project's objectives would not be met.

I dedicate this dissertation to all of the laboratory assistants, especially Puan Norsyamimi Farhana, Cik Wan Salmah Binti Wan Zalati, and Cik Amalina, for their assistance with all of my laboratory work and advice. It was a significant step toward the completion of this project.

I also dedicate this dissertation to my friends, who directly or indirectly helped me throughout the whole process. I would like to thank Chin Yi Lin, Tan Bee Yann, Yamunasri, and Alia for their unconditional encouragement and support.

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List of Abbreviations

AMR	Antimicrobial Resistance
CDC	Centers for Disease Control and Prevention
C. erzurumensis	*Calidifontibacillus erzurumensis*
DNA	Deoxyribonucleic acid
ESBL	Extended-Spectrum Beta-Lactamases
ESBL-EC	Extended-Spectrum Beta- Lactamase-producing *Escherichia coli*
HAI	Health-care-associated infections
HGT	Horizontal gene transfer
IDSA	Infectious Diseases Society of America
MCA	Mycological agar
MDR	Multidrug-Resistance
MHA	Mueller-Hinton Agar
MRSA	Methicillin-Resistance *Staphylococcus aureus*
NA	Nutrient Agar
NAOH	Sodium Hydroxide
PCR	Polymerase Chain Reaction
SDA	Sabouraud Dextrose Agar
VRE	vancomycin-resistant *enterococci*
WHO	World Health Organization
ZOI	Zone of Inhibition

ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, University Malaysia Kelantan, in partial requirement on the course DVT55204 - Research Project.

Antimicrobial resistance (AMR) is a public health issue in all regions, threatening both human and animal health. Finding and developing new antimicrobial drugs is critical for saving countless lives. Endophytes, a type of microorganism, act as reservoirs for bioactive metabolites with anticancer, antimicrobial, anti-inflammatory, anti-fungal, anti-viral, and other properties, which has makes it an ideal candidate for natural product production and piqued the interest of many researchers looking for new antimicrobial drugs. Hence, this study was conducted to isolate and identify endophytic fungi and bacteria from *Ipomoea aquatica* (water spinach), *Manihot esculenta* (cassava), and *Coleus aromaticus* (Mexican mint), and determine their antibacterial activities on Extended-Spectrum Beta- Lactamase-producing *Escherichia coli* (ESBL-EC) and Methicillin-Resistant *Staphylococcus aureus* (MRSA), and compare the antimicrobial activity with selected commercial antibiotics. Antimicrobial activity of endophytic bacteria and fungi were tested by using an agar plug diffusion assay. A total of ten isolates of endophytic bacteria and three isolates of endophytic fungi showed antimicrobial properties against MRSA and ESBL-EC. Among these three plants, water spinach and cassava have the most potential endophytes. Four to five endophytes were able to be isolated from the roots and shoots of both plants. The endophytic bacteria showing antimicrobial activities are *Bacillus* spp. (*B. velezensis, B. clarus, B. wledmannii*), *Staphylococcus* spp. (*S. caprae, S. capitis, S. epidermidis*),

Lysinibaccillus spp. (*L. boronitolerans, L. macroides, L. pakistanensis*) and *Calidifontibacillus erzurumensis* while Fungus 0, Fungus 1 and Fungus 2 have shown antimicrobial activities toward ESBL-EC and MRSA. These results indicate that these isolated endophytes could be a source of antimicrobial compounds for further investigation.

Keywords: AMR, Endophyte, ESBL-EC, MRSA, Natural products

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus DVT55204 - Projek Penyelidikan.

Rintangan antimikrob adalah isu kesihatan awam di semua wilayah, mengancam kesihatan manusia dan haiwan. Penemuan ubat antimikrob baharu adalah penting untuk menyelamatkan banyak nyawa. Endofit, sejenis mikroorganisma, bertindak sebagai sumber metabolit bioaktif dengan sifat-sifat antikanser, antimikrobial, anti-radang, anti-kulat, anti-virus, dan lain-lain, telah menjadikannya calon yang ideal untuk pengeluaran produk semula jadi dan menarik minat ramai penyelidik yang sedang mencari ubat antimikrob baharu. Oleh itu, kajian ini dijalankan untuk mengasingkan dan mengenal pasti aktiviti antibakteria kulat dan bakteria daripada *Ipomoea aquatica* (bayam air), *Manihot esculenta* (ubi kayu), dan *Coleus aromaticus* (pudina), terutamanya pada *Escherichia coli* penghasil Extended-Spectrum Beta-Lactamase (ESBL-EC) dan Methicillin-Resistant *Staphylococcus aureus* (MRSA), dan bandingkan aktiviti antimikrob dengan antibiotik komersial terpilih. Sebanyak sepuluh pencilan bacteria endofit dan tiga pencilan kulat endofit menunjukkan sifat antimikrob terhadap MRSA and ESBL-EC. Di antara ketiga-tiga tumbuhan, bayam dan ubi kayu mempunyai potensi endofit yang paling tinggi. Empat hingga lima endofit yang menunjukkan sifat antimikrob dapat diasingkan daripada kedua-dua tumbuhan. Beberapa bakteria endofit dan kulat yang terpencil menunjukkan aktiviti antibakteria terhadap ESBL-EC dan MRSA. Keputusan molekul menunjukkan bahawa bakteria endofit yang menunjukkan aktiviti antimikrob adalah *Bacillus* spp. (B. velezensis, B.

clarus, B. wledmannii), *Staphylococcus* spp. (S. caprae, S. capitis, S. epidermidis), *Lysinibaccillus* spp. (L. boronitolerans, L. macroides, L. pakistanensis) dan *Calidifontibacillus* erzurumensis. Keputusan ini menunjukkan bahawa endofit terpencil ini boleh menjadi sumber sebatian antimikrob untuk penyiasatan tambahan.

Kata Kunci: AMR, Endofit, ESBL-EC, MRSA, Produk semula jadi

1.0 INTRODUCTION

The natural evolutionary processes of the organism have led to biodiversity. The constant interactions between microorganisms and their environment over a long period of time can result in biological diversity and evolution as the organisms repeatedly adapt to the environment. As a result, the various types of environments promote a diversity of organisms that can adapt to them. Although many evolutionary events go unnoticed by us, the adaptation of microorganisms to antimicrobial drugs has become one of the most pressing global health issues, contributing to high human morbidity and mortality. Overexposure of microbes to different classes of antimicrobials has created life-threatening superbugs as they develop resistance to multiple drugs, leading to antimicrobial resistance (AMR). In other words, antibiotics are becoming more powerless against drug-resistant bacteria. AMR is defined as microorganism resistance to an antimicrobial to which they were previously sensitive (Jindal *et al*., 2015). The main routes for AMR transmission are agriculture, livestock rearing, the fishing industry, and poor infection control practices. AMR causes treatment delays, makes patients infectious for longer periods of time, raises the cost of treating common infections, and increases the risk of spreading resistant microorganisms to others (Prestinaci *et al*., 2015). If proper antimicrobial resistance control measures are not implemented, the looming fear of reaching a therapeutic dead end will become a reality as infectious diseases become more complex and, in some cases, impossible to treat. Because AMR poses a significant threat to individual patients, healthcare providers, the pharmaceutical industry, and society as a whole, a concerted effort and steps should be taken at all levels of the organization to mitigate the impact and prevent resistance from spreading further.

Because of the rising prevalence of antibiotic resistance among the most common pathogens in humans, discovering a new antimicrobial drug from a natural source is critical, as we need effective drugs to combat the superbug. Endophyte are a fascinating group of host-associated microorganism that frequently colonise the extracellular or intracellular tissue of the host, offer the host several advantages, and do not manifest any disease in the plant, have recently received a lot of attention because of their potential pharmacological properties (Alam *et al*., 2021). Endophyte can produce a wide range of bioactive metabolic compounds, including phenolic acids, alkaloids, quinones, steroids, saponins, tannins, and terpenoids, making them an excellent candidate for anticancer, antimalarial, antituberculosis, antiviral, anti-diabetic, anti-inflammatory, anti-arthritic, and immunosuppressive properties (Fadiji *et al*., 2020). The demonstration of these biological properties has attracted much research interest as they can be used in new drug inventions.

According to the research conducted by Hazalin *et al.* (2009), 28 out of 300 isolated endophytes exhibited antibacterial activity against at least one test microorganism with inhibition zones of 7 to 8 mm, and roughly half of the isolates exhibited inhibitory activity against *Escherichia coli.* A similar study was also conducted on *Coleus amboinicus,* a medicinal plant traditionally used to treat various diseases such as respiratory problems and digestive problems, and two out of three endophytic fungi with active biocompound production such as terpenoids, propylpropanoids, alkaloids, and phenylpropanoid compounds showed promising antibacterial potential through the agar plug diffusion method. These data strongly suggest the potential of endophytes isolated from plants as a source of antimicrobial agents (Astuti *et al*., 2014).

Approximately one hundred years ago, Malaysia was almost entirely covered by terrestrial forest and wetland forest, exhibiting a great diversity of ecosystems. Malaysia's vast flora diversity, combined with diverse chemical production, makes it an ideal candidate for natural product production. Furthermore, because Malaysia is a tropical country, most plants can grow all year. This increases our likelihood of isolating endophytes from diverse plant species. In this study, *Ipomoea aquatica* (water spinach), *Manihot esculenta* (cassava), and *Coleus aromaticus* (Mexican mint) were selected as samples because they can withstand harsh climatic conditions and are commonly found along roadways. Endophytic bacteria and fungi associated with *Ipomoea aquatica* (water spinach), *Manihot esculenta* (cassava), and *Coleus aromaticus* (Mexican mint) will be evaluated for their antimicrobial activity against a variety of test pathogens, particularly Extended-Spectrum Beta- Lactamase-producing *Escherichia coli* (ESBL-EC) and Methicillin-Resistant *Staphylococcus aureus* (MRSA).

1.1 RESEARCH PROBLEM STATEMENT

Because of antibiotic abuse, many bacteria have developed resistance to a wide range of antimicrobial drugs. The increased number of antimicrobial-resistant bacteria has caused serious problems because treatment options will be limited, increasing morbidity and mortality with an unexpected clinical outcome that will cost individuals and society more money. Both ESBL-EC and MRSA are the common healthcare-acquired infection (HAI) superbugs which not only threaten the patients' health and life but also bring additional economic burden to the patients and healthcare system by prolong the hospitalization. One approach to addressing this issue is to investigate natural products with antimicrobial properties as alternatives to existing antibiotics. Endophyte are one of the potential microorganisms that can help to solve this problem due to their potential antimicrobial properties. As a result, this study was conducted to isolate and identify endophytes from *Ipomoea aquatica* (water spinach), *Manihot esculenta* (cassava), and *Coleus aromaticus* (Mexican mint), determine their antibacterial properties against ESBL-EC and MRSA, and compare their antimicrobial effects with selected commercial antibiotics.

1.2 RESEARCH QUESTIONS

1. What endophytic fungi and bacteria can be isolated from the shoots and roots of *Ipomoea aquatica, Manihot esculenta,* and *Coleus aromaticus*?

2. Do endophytic fungi and endophytic bacteria isolated from the shoots and roots of *Ipomoea aquatica, Manihot esculenta,* and *Coleus aromaticus* have antimicrobial properties towards the ESBL-EC and MRSA?

3. Are the antimicrobial properties of endophytic fungi and endophytic bacteria isolated from the shoots and roots of *Ipomoea aquatica, Manihot esculenta,* and *Coleus aromaticus* more effective than selected commercial antibiotics?

1.3 RESEARCH HYPOTHESIS-what is the hypothesis for question 1?

1. Endophytic fungi and bacteria from the shoots and roots of *Ipomoea aquatica, Manihot esculenta,* and *Coleus aromaticus* have antimicrobial properties against ESBL-EC and MRSA.

2. Endophytic fungi and bacteria from the shoots and roots of *Ipomoea aquatica, Manihot esculenta,* and *Coleus aromaticus* are more effective than the selected commercial antibiotics regarding antimicrobial properties.

1.4 OBJECTIVES

1. To isolate and identify the endophytic fungi and bacteria from the shoots and roots of *Ipomoea aquatica, Manihot esculenta,* and *Coleus aromaticus*.

2. To determine the antimicrobial properties of endophytic fungi and bacteria isolated from the shoots and roots of *Ipomoea aquatica, Manihot esculenta,* and *Coleus aromaticus on* ESBL-EC and MRSA.

3. To compare the antimicrobial effect of endophytic fungi and bacteria isolated from the shoots and roots of *Ipomoea aquatica, Manihot esculenta,* and *Coleus aromaticus* with selected commercial antibiotics.

2.0 LITERATURE REVIEW

2.1 Antimicrobial Resistance

Antimicrobial resistance (AMR) is a natural process that takes place when microbes evolve mechanisms to protect themselves from antimicrobial effects. Antibiotics kill or inhibit susceptible bacteria, whereas bacteria that are naturally resistant or have acquired antibiotic-resistant traits have a better chance of surviving and multiplying. AMR is increased not only by overuse, but also by inappropriate use, such as poor dosing and noncompliance with treatment guidelines. This problem exists because bacteria have remarkable genetic plasticity, allowing them to respond to a wide range of environmental threats, including the presence of antibiotic molecules that can kill bacteria. Mutation in genes and acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT) will occur in bacteria to help them develop resistance toward antibiotics to which they are initially sensitive in order for them to adapt to the antibiotic, which may jeopardise their existence (Munita & Arias, 2016). Overuse of antibiotics, inappropriate antibiotic prescribing, and extensive use in agriculture, particularly in the livestock industry, are the leading causes of antibiotic resistance (Ventola *et al*., 2015).

Infections caused by AMR kill millions of people each year because resistant microbes are more difficult to treat, necessitate higher doses of antimicrobial drugs, or necessitate alternative medications that may be more toxic. If AMR did not exist, many lives could have been saved. Controlling AMR requires proper infection control, infection-specific prevention protocols, new antibiotic development, and management strategies. Another effective way to reduce AMR is through the controlled use of antimicrobials in

livestock. Furthermore, all patients, children, the general public, and relevant healthcare professionals must be educated on the unique characteristics of bacterial infections and antibiotics in order to raise AMR awareness (Lee *et al*., 2013). Antibiotics should be taken only when prescribed to avoid misuse or overuse, and narrow-spectrum antibiotics are preferred over broad-spectrum antibiotics when possible, as effectively and accurately targeting specific organisms is less likely to cause resistance as a side effect (Gerber *et al.*, 2017). People who take these medications at home must be educated on how to use them correctly. Health care providers can help to reduce the spread of resistant infections by using adequate sanitation and practising good hygiene, such as handwashing and disinfecting between patients, and they should encourage patients, visitors, and family members to do so as well.

2.2 Antimicrobial Resistance in Humans and Animals

The use, misuse, or overuse of antibiotics in clinical medicine is a major contributor to the development of AMR in human populations. Furthermore, as demand for animal protein has increased, the AMR crisis has spread to the food industry, particularly in livestock. Many studies have been conducted to investigate the relationship between antimicrobial use in animals and resistance in humans, involving both direct and indirect routes of transmission, according to Landers *et al*. The European Centre for Disease Prevention and Control, the European Food Safety Authority, and the European Medicines Agency conducted an aggregated analysis of surveillance data from across the Europe in 2015, assessing the relationship between antibiotic use in humans and animals and AMR. They reported that studies found a link between antimicrobial consumption and bacterial resistance in both humans and animals. Some studies also

discovered a link between antimicrobial consumption in animals and bacterial resistance in humans. This is due to the fact that antibiotics are commonly added to animal feeds as a growth promoter and a preventative measure to reduce the likelihood of infections. Because the bacteria are constantly exposed to antibiotics, which create selective pressure, this can result in a significant increase in the development of antimicrobial resistance in bacteria toward commercial antibiotics. When these animals are slaughtered and processed for food, pathogenic bacteria can contaminate meat or other animal products and spread to humans and the environment via the food chain and animal waste, respectively (Manyi *et al*., 2018). Contaminated fruits and vegetables, for example, can be caused by contact with contaminated soil, water, or fertiliser. Antibiotic-resistant intestinal infections can be contracted by handling or eating contaminated food or coming into contact with animal waste. Infected people have the potential to spread the resistant bacteria to others through community or nosocomial spread, resulting in a significant increase in the population at risk and, as a result, an increase in the number of infections caused by multidrug-resistant bacteria (Duin *et al.*, 2016).

2.3 Bacteria infections caused by Resistant Bacteria

Antibiotic-resistant infections are already common around the world. The Infectious Diseases Society of America (IDSA) Emerging Infections Network conducted a national survey of infectious-disease specialists in 2011 and discovered that more than 60% of participants had seen a pan-resistant, untreatable bacterial infection in the previous year (Spellberg *et al*., 2014). The rapid emergence of resistant bacteria has been described by many public health organizations as a "crisis" or "nightmare

scenario" with "catastrophic consequences." The Centers for Disease Control and Prevention (CDC) declared in 2013 that the human race has entered the "post-antibiotic era," and the World Health Organization (WHO) warned in 2014 that the antibiotic resistance crisis is becoming dire.

A global pandemic of resistant *Staphylococcus aureus* and *Enterococcus* species is currently the most dangerous gram-positive pathogen. Every year, Methicillin-Resistance *S. aureus* kills more Americans than HIV/AIDS, Parkinson's disease, emphysema, and homicide combined (Gross M, 2013). Many common antibiotics are no longer effective against vancomycin-resistant *enterococci* (VRE) and a growing number of other pathogens. It is a worldwide epidemic that common respiratory pathogens such as *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* are becoming drug resistant.

Gram-negative pathogens are particularly concerning because they are becoming resistant to nearly all antibiotic drug options, eerily reminiscent of the pre-antibiotic era (Rossolini *et al.,* 2014). The emergence of Multidrug-Resistance (MDR) and increasingly pan-resistant gram-negative bacilli has had an impact on medical practice in every field. The most serious gram-negative infections happen in hospitals are caused by Enterobacteriaceae mostly *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. MDR gram-negative pathogens are also on the rise in the community. These include *Escherichia coli* and *Neisseria gonorrhoeae,* both of which produce Extended-Spectrum Beta-Lactamase (ESBL).

The CDC evaluated antibiotic-resistant bacterial infections based on seven criteria which are clinical impact, economic impact, incidence, a 10-year projection of incidence, transmissibility, availability of effective antibiotics, and prevention barriers. Gross, M. (2013) classified each bacteria's threat level as "urgent," "serious," or "concerning" based on its risk to human health. Both the bacteria tested in this study, ESBL-EC and MRSA, are under serious threat and necessarily require increased monitoring and prevention efforts.

2.4 Methicillin-Resistance *Staphylococcus aurues* (MRSA) and Extended-Spectrum Beta-Lactamase- Producing *Escherichia coli* (ESBL-EC)

Since the discovery of MRSA five decades ago, it has spread throughout the world, with a high prevalence in several countries in Europe, the Americas, and the Asia-Pacific region (Rossolini *et al*., 2014). MRSA infections can be fatal, and they are among the most common antibiotic-resistant threats. According to Gross (2013), MRSA is responsible for 11,285 deaths in the United States each year.

Beta-lactam antibiotics that are similar to penicillin are the antibiotics that MRSA resistant to. However, glycopeptides such as vancomycin and teicoplanin, linezolid, tigecycline, daptomycin, and even some new beta-lactams, such as ceftaroline and ceftobiprole, continue to be active against MRSA. MRSA, on the other hand, has demonstrated remarkable adaptability in emerging and spreading in a variety of epidemiological settings over time such as in hospitals, the community, and, more recently, in animals. This complicates the epidemiology of MRSA infections and poses a challenge to infection-control systems that are solely focused on health-care-

associated infections (HAIs) (Rossolini *et al.*, 2014). Furthermore, while resistance to anti-MRSA agents typically occurs through bacterial mutation, there have been reports of resistance to linezolid and glycopeptide antibiotics being transferred, which is cause for serious concern.

Fortunately, the incidence of HAI-MRSA infections appears to be decreasing as a result of aggressive preventive hygiene measures in hospitals in some areas such as the Netherlands and the United Kingdom (Rossolini *et al*., 2014). Between 2005 and 2011, overall rates of invasive MRSA decreased by 31%, with HAIs experiencing the greatest decreases (around 54%). This finding demonstrates that infection control can be extremely effective in limiting MRSA spread. However, according to a report from the CDC in the United States, rates of community-acquired MRSA infections among the general population have increased rapidly over the last decade. While there is evidence that these increases are slowing, they are not following the same downward trends as hospital-acquired MRSA infections.

A broad-spectrum beta-lactamase enzyme was carried by ESBL-EC which enables them to become resistant to a wide variety of penicillin and cephalosporin antibiotics. According to CDC, Office of Infectious Disease Antibiotic resistance threats in the United States, ESBL-EC cause 26,000 HAIs and 1,700 deaths per year. Some ESBL-EC are resistant to nearly all antibiotics in the penicillin and cephalosporin classes. In such cases, the remaining treatment option is an antibiotic from the carbapenem family. However, these drugs should be used with caution since use contributes to resistance.

2.5 Endophytic Fungi and Endophytic Bacteria as a source of alternative antimicrobial agents

An endophyte is a plant-inhabiting organism. The organisms are a group of endosymbiotic microorganisms that are associated with plants in various forms, including bacteria (actinomycetes or mycoplasma) or fungi that have colonised the plant tissues (Wilson, 1995). More than 200 genera from 16 phyla of bacterial species have been reported to be associated with endophytes, with the majority belonging to Actinobacteria, Proteobacteria, and Firmicutes (Golinska *et al*., 2015). Endophytic bacteria range from gram-positive to gram-negative organisms, including *Achromobacter, Acinetobacter, Agrobacterium, Bacillus, Brevibacterium, Microbacterium, Pseudomonas, Xanthomonas,* and the list goes on (Sun *et al*., 2013). Bacterial endophytes are diverse in nature and are known to produce a variety of bioactive metabolites, such as antimicrobial and anticancer compounds, with 76% of them reported from a single genus, *Streptomyces* (Berdy, 2012). They serve as repositories of novel bioactive secondary metabolites, such as alkaloids, phenolic acids, quinones, steroids, saponins, tannins, and terpenoids, which have antimicrobial, anti-insect, and anti-cancer properties, and many others. This suggests that endophytes may be a source of novel antibiotics. In the absence of a viable alternative, the emergence of antimicrobial resistance necessitates the constant development of new antibiotics. Consequently, these endophytes, which reside within plants, are increasingly become the focus of research, particularly when the plant is traditionally used for medicinal purposes. In 2010, Janso and Carter were able to isolate endophytes from native tropical Papua-New Guinea and Mborokua Island plants (Solomon Islands). A total number of 105 different strains were isolated, and their 16S rRNA sequences were used to classify

them into 17 genera, some of which were proposed as new genera. Nearly half of the endophyte extracts displayed bioactivity against MRSA, *Candida albicans*, and *E. coli*.

3.0 MATERIAL AND METHODS

3.1 Source of Endophytic Fungi and Endophytic Bacteria

The source of endophytic fungus in this scenario will be a plant that is typically present in the local environment, has the capacity to withstand harsh climatic conditions, and is persistent under trampling. The three plants *Manihot esculenta* (cassava) (Figure 1), *Ipomoea aquatica (*water spinach) (Figure 2), and *Coleus aromaticus* (Mexican mint) (Figure 3) were chosen as samples for this investigation. The plants chosen for this study were found in Kelantan at neighborhood, stores or by the side of the road. In order to reduce moisture and hasten drying, plants with healthy-appearing, fresh, and crisp branches and roots were picked and kept in the newspaper. To prevent contamination, the pieces and newspaper were kept together in a clean plastic bag. Once collection was complete, the samples were brought to the Bacteriology Laboratory at the Faculty of Veterinary Medicine, University Malaysia Kelantan, where they were isolated for endophytic fungi and bacteria.

Figure 1. *Ipomoea aquatica* (water spinach)

Figure 2. ***Manihot esculenta*** **(cassava)**

Figure 3. ***Coleus aromaticus*** **(Mexican mint)**

3.2 Isolation and Identification of Endophytic Fungi and Endophytic Bacteria

Isolation and identification of endophytic fungi and endophytic bacteria were carried out following Liang *et al*. (2012) with some modifications. First, the samples were rinsed with running water to remove the surface dirt, followed by the immersion of the pieces in 75 percent ethanol for 1 minute and 2% NaOH for 5 minutes. After that, the samples were washed with sterile distilled water three times. After that, the samples were put on sterilised filter paper to allow the surface of the sample to become dry. Finally, the samples were cut into smaller pieces and crushed with a mortar and pestle. The crushed samples were placed on Sabouraud Dextrose Agar (SDA) and incubated for 1-2 weeks (Figure 4). The plates were observed daily for any bacterial and fungal growth. Identification and characterization of the bacteria and fungi were done based on the morphology of the colonies, including the colony topography, color, and growth pattern of the colonies, and under microscopic examination. To obtain pure cultures, the bacterial and fungal colonies were picked using a sterile needle tip and subcultured onto SDA (Himedia, India) devoid of antibiotics for the fungal colonies, while the bacterial colonies were subcultured on Nutrient Agar (NA) (Himedia, India). Further confirmation of the fungus and bacteria species was done using polymerase chain reaction (PCR) amplification and subsequent sequencing.

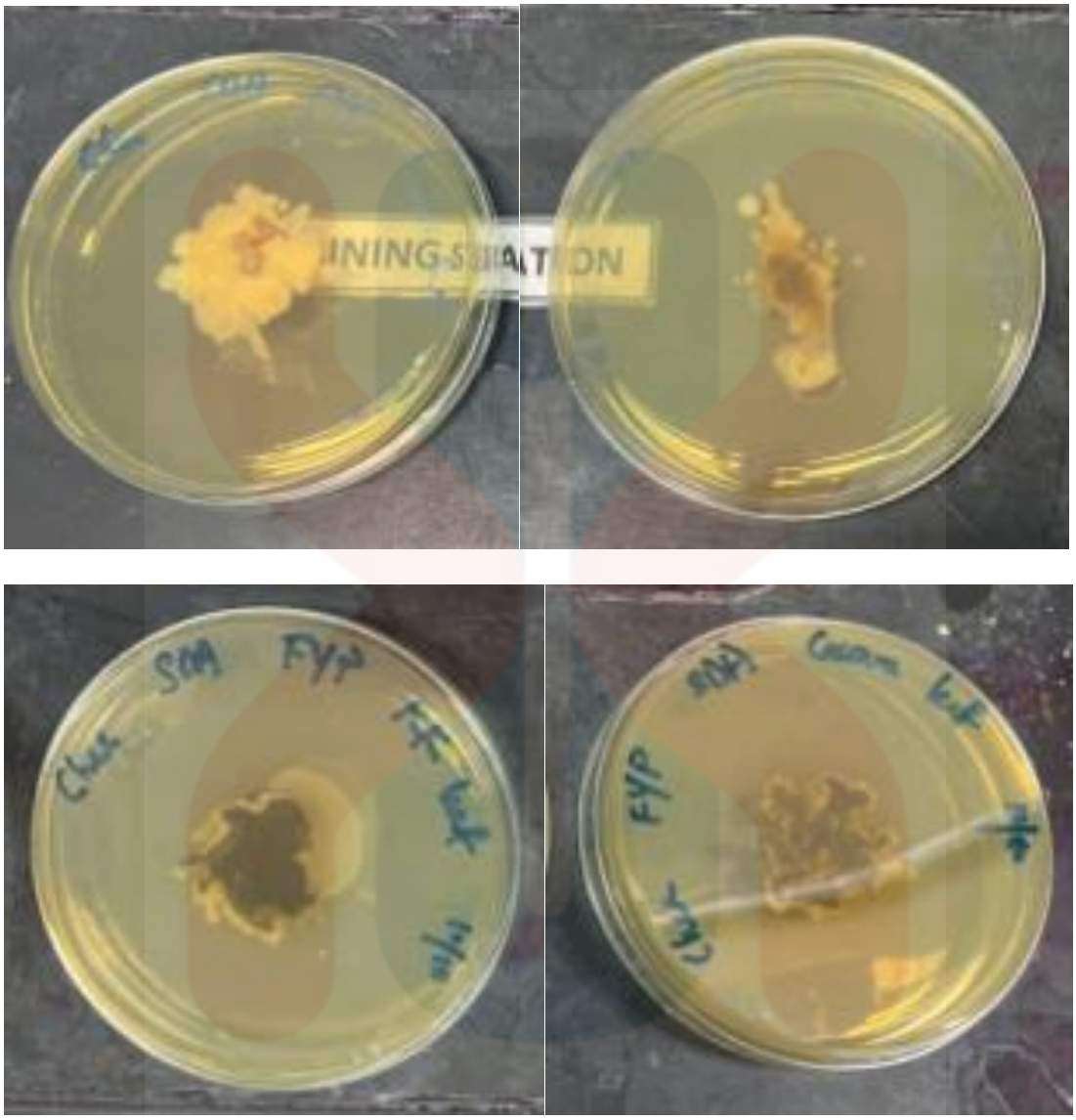

Figure 4. The leaves and roots of the samples were crushed and placed on SDA agar.

3.3 Deoxyribonucleic acid (DNA) Extraction

Deoxyribonucleic acid (DNA) extraction of endophytic fungi and bacteria was carried out using the Geneaid® Blood/Cultured Cell DNA Extraction Kit and Geneaid® Tissue DNA Extraction Kit (Geneaid, Taiwan), respectively. The DNA extraction was done by following the manufacturer's recommendation with some modifications.

To perform bacterial DNA extraction, a sterile inoculating loop was used to harvest the culture as a sample and mix it with 1 ml of sterile distilled water in a 1.5 ml sterile microcentrifuge tube. The bacterial samples were vortexed gently to get a homogenised solution. 300µL GBT buffer and 30µL proteinase K were added to the samples. The mixtures were vortexed again to get a homogenised solution, followed by incubation at 60^{o}C for overnight. After overnight incubation, the mixture was added to 200 L absolute ethanol and vigorously shaken for 10 minutes before being transferred to the GD column. The rest of the steps were the same as those stated in the support protocol. For fungal DNA extraction, a loopful of fungus was harvested and mixed with 1 mL of sterile distilled water. After that, the mixtures were vortexed vigorously to homogenise the solutions. The mixtures were then incubated at -20^{o}C for 1 hour. After a period of cold shock, the mixtures were vigorously vortexed before being incubated in a water bath at 60 ^{o}C for 30 minutes. Following that, the mixtures were centrifuged at 5,000 x g for 5 minutes. 200 µL GB buffer was added to the sample, and the tube was shaken vigorously. The rest of the steps stated in the provided fungus protocol were followed.

3.4 Determination of Concentration and Purity of extracted DNA

To measure the concentration and purity of the extracted DNA, a Nanophotometer (Nanodrop Technologies, Wilmington) was used. Firstly, 1 ul of AE buffer was used as a blanking solution to calibrate the instrument before measuring the DNA concentration. Next, 1µL of DNA sample was measured at 260 nm wave length and the result was read in ng/ul while the ratio of absorbance at 260 and 280 nm was used to determine the purity of the DNA sample. The ideal purity range should be 1.7–2.0 at

OD260/OD280. After checking the DNA concentration, all the samples were stored at -20 ^{o}C in the aquatic laboratory for further use.

3.5 Polymerase Chain Reaction (PCR) Amplification of ITS and EF Genes for Identification of Fungi Species

Amplification of the ITS and EF genes to identify fungi species was performed by using C1000 TouchTM 96-well Thermal Cyclers (Bio-Rad Laboratories, Foster City, CA). The initial PCR reaction before optimization of PCR assay includes: 25 ul 1x Green GoTaq Green Master Mix, 1uM of each forward and reverse primer set, 3–10 ul DNA template, and 13–17 ul Nuclease Free Water (NFW). The final volume is 50 ul, and a negative control containing NFW will be included in each PCR assay.

The two sets of published universal primers for fungus used in PCR amplification with their cycling conditions are listed in Table 1 below:

Table 1. Oligonucleotide sequences used for PCR identification of fungi species and the respective cycling conditions.

No.	Name	Gene Coverage	References
1.	ITS1/ ITS4	Forward: 5'-TCCGTAGGTGAACCTGCGG-3' Reverse: 5'-TCCTCCGTTGATATGC-3'	(Maadon *et al.*, 2018)
		Cycling condition	

		● One cycle for initial denaturation (98°C for 2 min); ● 25 cycles for denaturation, annealing, and extension - 98°C for 15 s for denaturation; - 60°C for 30 s for annealing; and - 72°C for 30 s for extension for extension, ● One cycle (72°C for 10 min) for final extension of the amplified DNA	
2.	EF1-1018F / EF1-1620R	Forward: 5'-GAYTTCATCAAGAACATGAT-3' Reverse: 5'-GACGTTGAADCCRACRTTGTC-3' **Cycling condition** ● One cycle for initial denaturation at 95 °C for 5 min, ● 35 cycles for denaturation, annealing, and extension - 94 °C for 30 s for denaturation, - 52 °C for 30 s for annealing, and - 72 °C for 1 min for extension ● One cycle for final extension step at 72 °C for 8 min.	(Raja *et al*., 2017)

3.6 Polymerase Chain Reaction (PCR) Amplification of 16s Ribosomal RNA Genes for Identification of Bacterial Species

Amplification of the genes to identify fungi species was performed by using C1000 TouchTM 96-well Thermal Cyclers (Bio-Rad Laboratories, Foster City, CA). Before optimising the PCR assay, 25 ul of 1x Green GoTaq Green Master Mix, 1 uM of each forward and reverse primer set, 3–10 ul of DNA template, and 13–17 ul of NFW are used in the initial PCR reaction. The final volume is 50 ul, and a negative control containing NFW will be included in each PCR assay.

One set of published universal primers for bacteria was used in PCR amplification, and their cycling conditions are listed in Table 2 below:

Table 2. Oligonucleotide sequences used for PCR identification of fungi species and the cycling conditions.

No.	Name	Gene Coverage	References
1.	Taf/ Tar	Forward: 5′-TCCTACGGGAGGCAGCAGT-3′ Reverse:5′-GGACTACCAGGGTATCTAATCCTGTT-3′ **Cycling condition** ● One cycle for initial denaturation (94°C for 3min); ● 30 cycles for denaturation, annealing, and extension - 94°C for 45 s for denaturation; - 61°C for 1 s for annealing; and - 72°C for 3 min for extension for extension, ● One cycle (72°C for 5 min) for final extension of the amplified DNA	(Zucol *et al*., 2006)

3.7 Gel Electrophoresis

Five microliters of each PCR product were inserted into the well of a 1.5% agarose gel by using a micropipette, and the gel was immersed in the TBE buffer. Electrophoresis was carried out for 40 minutes at 100 volts and 400 mA. The gel was stained with Midori Green Advance DNA stain (Nippon Genetics, Europe) for better visualization, and the stained gel was visualised under a UV illuminator using Gel DocTM EZ Imager

(Biorad, USA). BenchTop 100 bp DNA ladder (Promega, USA) was used as the molecular weight marker.

3.8 Sequencing of PCR products

The PCR products, together with the forward primer and reverse primer, were sent to Apical Scientific Sdn Bhd, Selangor, for sequencing. The DNA sequences were analysed and trimmed using Bioedit software, and BLAST was done on the NCBI platform.

3.9 Test Microorganism

Extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-EC), *E. coli (ATCC 25922)*, MRSA, and *S. aureus* were obtained from the existing stock cultures maintained by Dr. Erkihun's research team at the Zoonotic Diseases and Public Health Research Laboratory, Faculty of Veterinary Medicine, University Malaysia Kelantan. The cultures were refreshed on NA, followed by culturing on differential media such as Mac Conkey agar for ESBL-EC *and* ATCC *E. coli* while Mannitol Salt Agar for MRSA and *S. aureus.* All the microbial cultures were adjusted to 0.9 McFarland standards, which will be visually comparable to a microbial suspension of approximately 1.5 x 10^8 CFU/mL.

3.10 Evaluation of Antibacterial Activity

Agar plugs diffusion was used to test the antibacterial effects of endophytic fungi and bacteria from *Ipomoea aquatica, Manihot esculenta,* and *Coleus aromaticus.* Test bacteria were inoculated on a Petri dish with Mueller-Hinton Agar (MHA) to form a bacteria lawn. To test the antimicrobial activity of isolated endophytic fungi and bacteria, an agar plug with a diameter of approximately 8 mm was cut from the SDA and NA plates of actively growing endophytes, respectively, and transferred to the MHA with the test bacteria. To determine the antimicrobial properties of endophytic bacteria, agar plugs with an 8-mm diameter were cut from the NA plate, which contained actively growing endophytic bacteria, and transferred to the MHA with the test bacteria. The same steps were repeated twice for two different replicates.

Parafilm was used to seal the inoculated plates. Table 3 shows the conditions under which the plates were stored.

Table 3: Two Different Conditions for Agar Plug Diffusion Assays

Type of Condition	Condition of incubation
Condition 1	chilled at 4^{o} C for 24 hours for t*he* diffusion of metabolites, then incubated in a*n* incubator at 37^{o} C for 24 hours.
Condition 2	incubated at 37 ^{o}C directly in the incubator f*or* 24 hours.

The diameters of zones of inhibition (ZOI) were measured in mm by using a ruler after incubation.

3.11 Comparison of Antibacterial Activity with Commercial Antibiotic

The antibacterial activities of the isolated endophytic fungi and endophytic bacteria from *Ipomoea aquatica*, *Manihot esculenta*, and *Coleus aromaticus* towards ESBL-EC, ATCC *E. coli*, *S. aureus*, and MRSA were compared with the antimicrobial effect of commercially available antibiotics. The test organisms ESBL-EC, *E. coli* (ATCC 25922), *S. aureus*, and MRSA were swabbed over the surface of sterile MHA agar plates using a sterile cotton swab. The antibiotic discs (ampicillin, amoxicillin, enrofloxacin, cephalexin, and trimethoprim) (Oxoid, United Kingdom) were placed on the surface of the plates. The plates containing antibiotic discs and test bacteria were incubated at 37^{o} C for 24 hours. After incubation, the diameter of the inhibition zones (mm) was measured using a ruler, and the results were recorded. The same steps were repeated twice for two replicates. The interpretation of the ZOI for the antibiotics was made according to the standard guidelines of the Clinical and Laboratory Standard Institute (CLSI, 2021).

4.0 RESULT

4.1 Isolation and Identification of Endophyte

After placing the plant samples on the Sabouraud Dextrose Agar (SDA), growths with different morphologies were able to be observed on the SDA agar plates. Morphologically, most of the growths on SDA agar with different plant samples showed rhizoid-like characteristics with dry, compact, whitish to creamy, raised wrinkles, and ramifications in a tree-like formation 7 days after incubation. There are also growths with a yellowish or beige color, ground glass appearance, and a smooth and compact colony with a lobate or curled margin observed on the plates as well. Besides, a white mold colony was able to be observed on the agar plate with cassava leaves, stems of Mexican mint, and water spinach leaves after 5 to 7 days of incubation (Figure 5, 6, 7, 8).

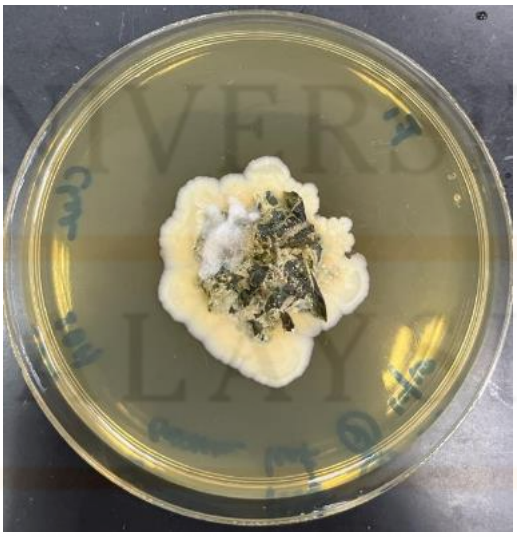

Figure 5: Two different growth patterns were observed on the SDA plate with Cassava leaves after 7 days of incubation. Moldy growth was observed on the sample, while an irregular, glistening, beige-colored colony with a lobate margin was observed at the edges of the sample.

Figure 6: Another plate with Cassava leaf samples that show growth with different morphology after 7 days of incubation. A rhizoid, veined, rough, white to cream-colored colony with lobate margins was observed on the plate. There is also white mold growing on the plant sample.

Figure 7: Growths with different types of morphology were shown on the SDA agar plate, which contained the stem of Mexican mint. Some growths show rhizoid, veined, rough, white to cream-colored colony with a lobate margin, while some growths show an irregular, raised, rough surface and are white or orange in color.

Figure 8: White filamentous, mouldy growth filling the petri dish was observed on the SDA agar plate with water spinach leaves 5 days after incubation.

Gram staining was done on the growth to examine and identify the endophytic bacteria isolated from the plant samples. Most of the growth shows Gram-positive rod-like organisms with or without spores, which are presumptively identified as *Bacillus spp*. A few colonies show gram-positive cocci and coccobacilli, while others show gram-negative rods (Figure 9, 10, 11, 12).

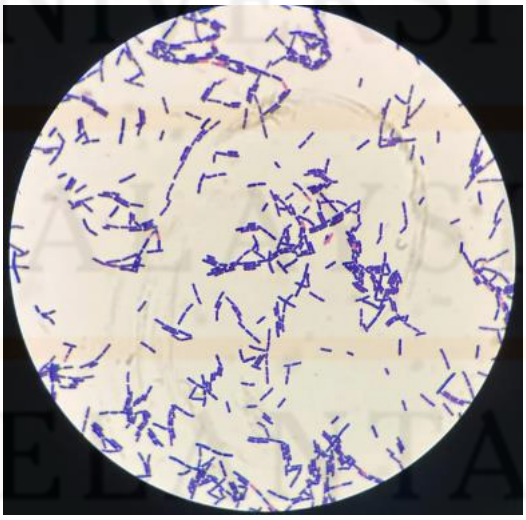

Figure 9: Gram-positive diplobacilli was observed.

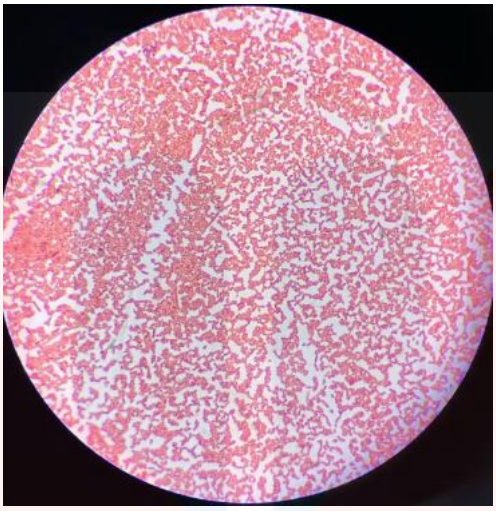

Figure 10: Gram-negative short rod and coccobacilli arranged in cluster was observed.

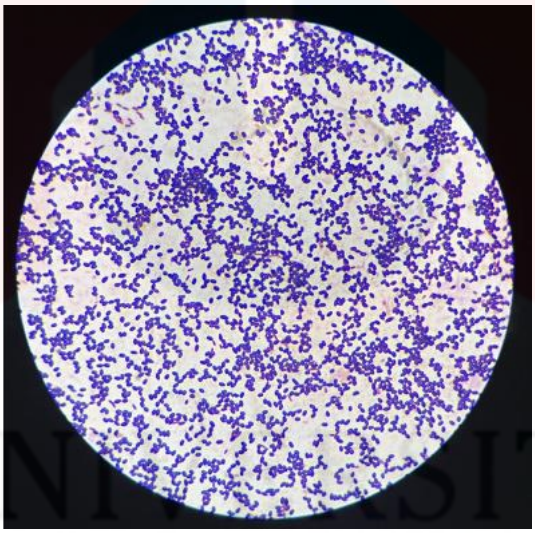

Figure 11: One of the growths isolated from water spinach leaves sample shows gram-positive coccobacilli or yeast-like organisms.

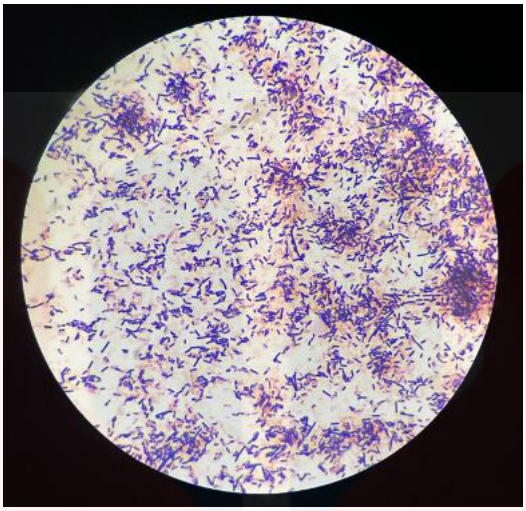

Figure 12: Another growth isolated from root of water spinach shows gram-positive bacilli with central spore.

The mouldy growths observed on the plant samples were subcultured onto a fresh SDA and incubated at 40ºC for another 7–14 days. Four different fungi were isolated from the plant sample. Some colonies have a woolly, cotton candy-like texture, while others have a dry, powdery, felt-like texture and a colour range ranging from white to greyish-black. Lactophenol cotton blue staining was done to observe the microscopic appearance of the fungus. (Figure 13,14,15,16)

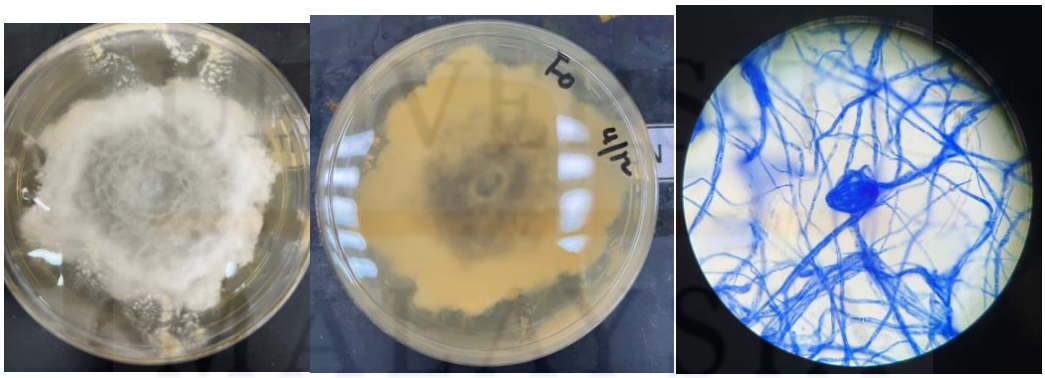

Figure 13: The left image shows the colony's macroscopic appearance, which is greyish-black in colour with a hairy or woolly texture resembling cotton candy filling the plate. The middle image shows the reverse of the colony, with a greyish- black colour in the centre and a cream to yellow border. Microscopic examination revealed septate hyaline hyphae with conidia in balls.

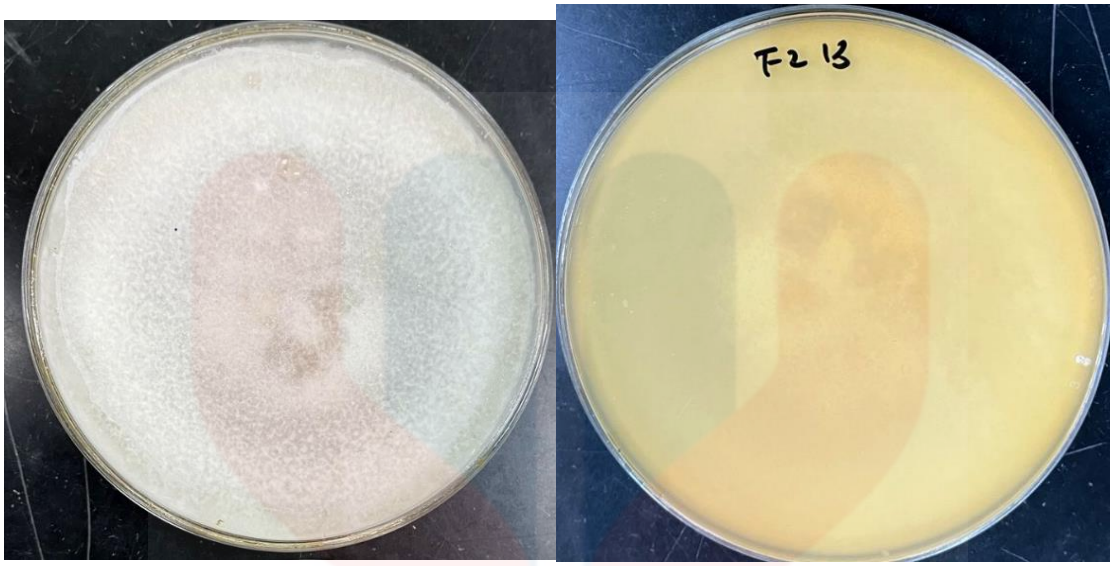

Figure 14: The colony showed a dry, felt-like surface texture with a powdery appearance over the SDA. The reverse shows white in color.

Figure 15: This colony is rather flat compared to other colonies. The colony appeared velvety to powdery in texture with folded, wrinkled, or cerebriform surface contours. The reverse was tan or light brown. Microscopically, hyaline septate hyphae with short cylindrical conidia are found along the sides of the conidiophore.

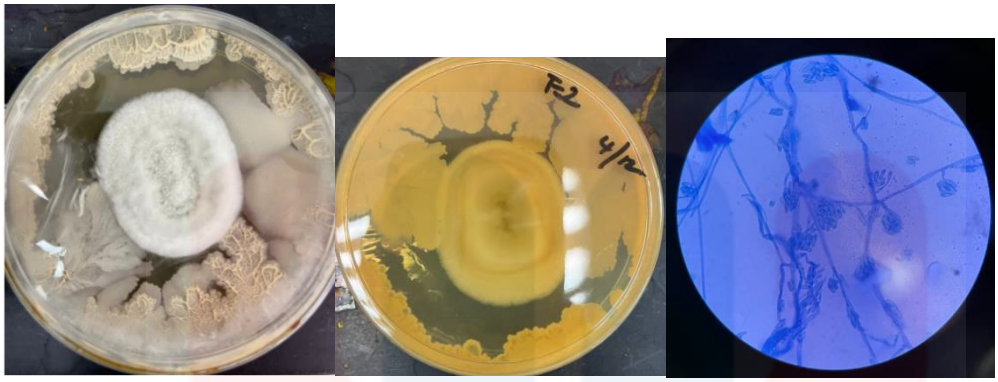

Figure 16: The colony has a woolly to cottony texture and is white in color, gradually turning grayish. The reverse is pale in colour with a greyish or black center. Microscopically, the hyphae appear hyaline and septate, while the conidia are cylindrical, accumulate along the sides of the conidiophore and hyphae.

4.2 In Vitro Antimicrobial Activity of Endophyte Isolated

The isolated endophyte was screened for its potential antibacterial activity against test bacteria by the agar plug diffusion method (Figure 24, 25). The Mueller-Hinton Agar (MHA) plate with an endophyte agar plug was incubated at different conditions for its metabolite's diffusion (Figure 17, 18, 19, 20). Most of the endophyte isolated inhibited at least one of the bacteria in the agar plug diffusion assay and created a zone of inhibition (ZOI) ranging from 10 mm to 50 mm (Table 4 and Table 5). The highest maximum inhibition zone created by the isolated endophyte against Methicillin-Resistance *Staphylococcus aureus* (MRSA) is 50 mm, while the highest maximum inhibition zone created by the isolated endophyte against Extended-Spectrum Beta-Lactamase-producing *Escherichia coli* (ESBL-EC) is 30 x 40 mm.

Antimicrobial activity against both ATCC *E. coli* and ESBL-EC is demonstrated by endophytic bacteria isolated from Mexican mint leaves (MML (A) 2), water spinach roots (KKR (A) 1, KKR (A) 2), cassava leaves (CL (B) 1), and Fungus 1 (F1). Endophytic bacteria from Mexican mint stem (MMS (A/F) 1), and endophytic bacteria

isolated from cassava leaves (CL (B) 2) only show antimicrobial activities toward ATCC *E. coli*, while Fungus 2 (F2) only shows antimicrobial activities toward ESBL-EC. Endophytic bacteria from Mexican mint leaves (MML(A)2), F1, and F2 show antibacterial activity against *S. aureus* and MRSA. Endophytic bacteria isolated from Cassava (CR(A)1, CLA(A) 1, CL(A) 2), endophytic bacteria from Mexican mint (MMS(B) 2), and the bacteria from water spinach (KKR (C) 1, KKL (B) 1, KKL (B) 2), and F0 all have ZOI against *S. aureus*, whereas the endophytic bacteria isolated from cassava (CL(B) 1, CL(B) 2), the endophytic bacterial isolated from Mexican mint (MML(A) 2) ,the endophytic bacterial isolated from water spinach (KKR (A)1, KKR (A)2) , and Fungus 0 (F0) only have antimicrobial activities against MRSA.

Table 4: ZOI (mm) of isolated Endophyte against selected test pathogens (ATCC *E. coli* & ESBL-EC)

Isolated Endophyte	Zone of Inhibition (mm)			
	ATCC. *E. coli*		ESBL-EC	
	direct incubate in incubator (37°C)	Incubate in chiller for 24 h before incubate in incubator	direct incubate in incubator (37°C)	Incubate in chiller for 24 h before incubate in incubator
CL (A) 1	-	-	-	-
CL (A) 2	-	-	-	-
MML (A) 1	-	-	-	-
MML (A) 2	-	25	-	15
MMS (A/F) 1	15	20	~	-
MMS (A/F) 2	-	-	-	-
MMS (B) 1	-	-	-	-
MMS (B) 2	-	-	-	-
KKR (A) 1	18	~	-	15
KKR (A) 2	18	~	-	-
KKR (C) 1	-	-	-	-
KKR (C) 2	-	-	-	-
KKL (A) 1	-	-	-	-
KKL (A) 2	-	-	-	-
KKL (A) 3 / P	-	-	-	-
KKL (B) 1	-	-	-	-
KKL (B) 2	-	-	-	-
CR (A) 1	-	-	-	-
CR (A) 2	-	-	-	-
CL (B) 1	18	18	~	25
CL (B) 2	18	18	-	-
F0	-	-	-	-
F1	11	20	~	15
F2	-	-	35	~
F3	-	-	-	-

~: antimicrobial properties without clear zone of inhibition
-: no zone of inhibition observed
CL (A), CL (B): endophytic bacterial isolated from Cassava Leaves
CR (A): endophytic bacteria isolated from Cassava Roots
KKR (A), KKR (C): endophytic bacteria isolated from water spinach roots.
KKL (A), KKL (B): endophytic bacteria isolated from water spinach leaves.
MML (A): endophytic bacteria isolated from Mexican mint leaves
MMS (A/F), MMS (B): endophytic bacteria isolated from Mexican mint roots
F: endophytic fungi isolated from the plant samples

Table 5: ZOI of isolated endophyte against selected test pathogens (*S. aureus* & MRSA)

Isolated Endophyte	Zone of Inhibition (mm)			
	S. aureus		MRSA	
	direct incubate in incubator	Incubate in chiller (4ºC) for 24 h before incubate in incubator	direct incubate in incubator	Incubate in chiller (4ºC) for 24 h before incubate in incubator
CR (A) 1	30	30	~	-
CR (A) 2	~	~	~	-
CL (B) 1	~	~	35	45
CL (B) 2	~	~	30	45
MMS (A/F) 1	~	-	-	-
MMS (A/F) 2	~	-	-	-
MMS (B) 1	~	-	-	-
MMS (B) 2	30	~	-	-
CL (A) 1	22	30	~	-
CL (A) 2	20	30	~	-
MML (A) 1	-	-	~	-
MML (A) 2	35	-	45	40
KKR (A) 1	~	~	35	50
KKR (A) 2	~	~	40	50
KKR (C) 1	23	23	-	-
KKR (C) 2	-	-	-	-
LB (1)	-	-	-	-
KKL (A) 1	-	-	-	-
KKL (A) 2	-	-	-	-
KKL (A) 3 / P	-	-	-	-
KKL (B) 1	40	45	-	-
KKL (B) 2	30	-	-	-
F0	-	-	25	35
F1	-	23	-	25
F2	16	~	~	20
F3	-	-	-	-

~: antimicrobial properties without clear zone of inhibition observed
-: no zone of inhibition observed
~: antimicrobial properties without clear zone of inhibition observed
-: no zone of inhibition observed
CL (A), CL (B): endophytic bacterial isolated from Cassava Leaves

CR (A): endophytic bacteria isolated from Cassava Roots
KKR (A), KKR (C): endophytic bacteria isolated from water spinach roots.
KKL (A), KKL (B): endophytic bacteria isolated from water spinach leaves.
MML (A): endophytic bacteria isolated from Mexican mint leaves
MMS (A/F), MMS (B): endophytic bacteria isolated from Mexican mint roots
F: endophytic fungi isolated from the plant samples

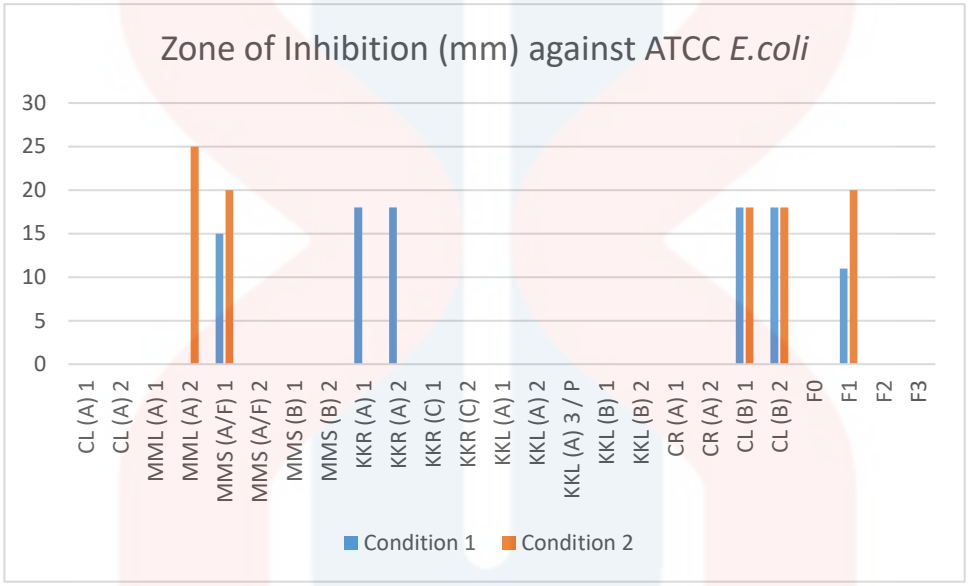

Figure 17: ZOI (mm) of isolated endophytes against ATCC *E. coli* at different condition where condition 1 = direct incubation at 37 ^{o}C, condition 2 = Prior incubation in fridge (4^{o}C) for 24 hours

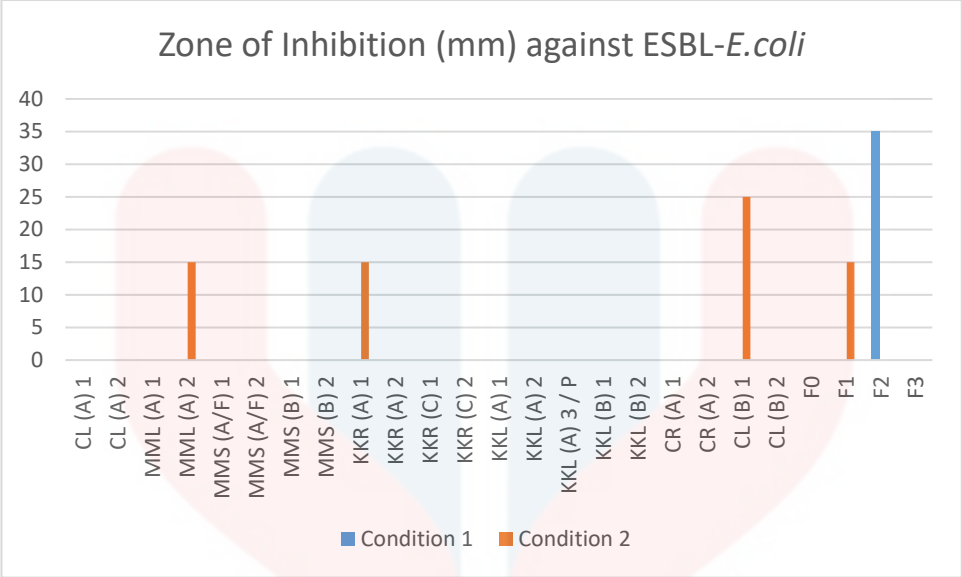

Figure 18: ZOI (mm) of isolated endophytes against ESBL- *E. coli* at different condition where condition 1 = direct incubation at 37 ^{o}C, condition 2 = Prior incubation in fridge (4^{o}C) for 24 hours

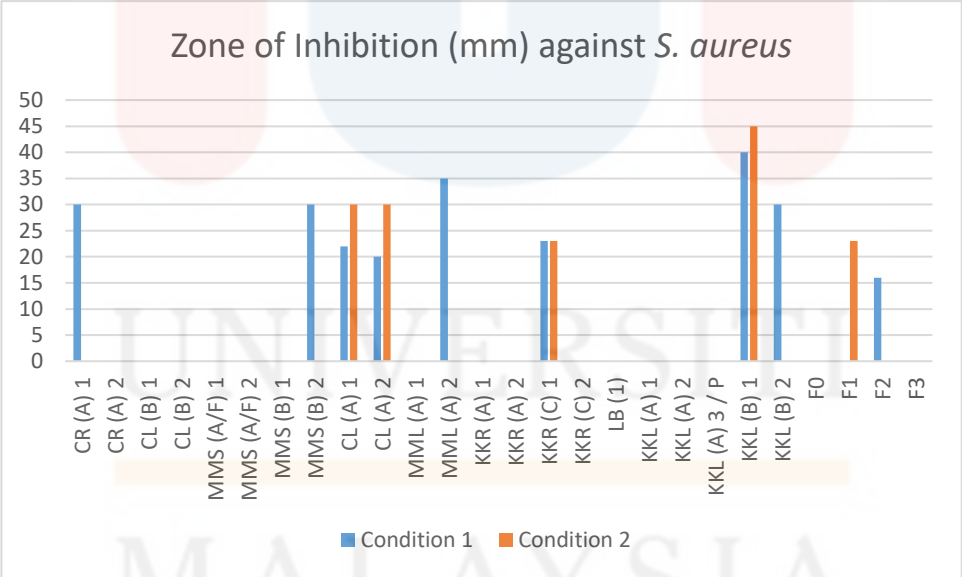

Figure 19: ZOI (mm) of isolated endophytes against *S. aureus* at different condition where condition 1 = direct incubation at 37^{o}C, condition 2 = Prior incubation in fridge (4^{o}C) for 24 hours

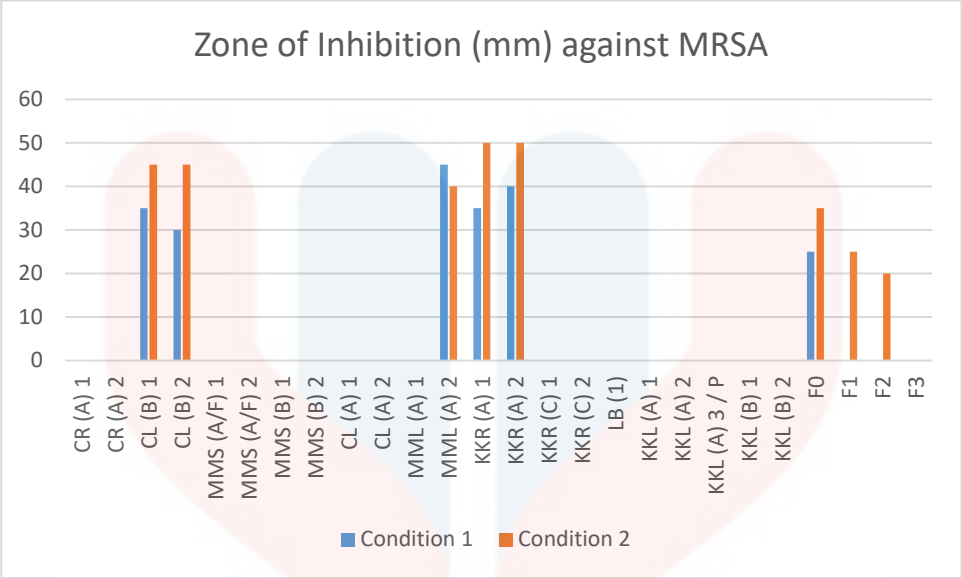

Figure 20: ZOI (mm) of isolated endophytes against MRSA at different condition where condition 1 = direct incubation at 37 ^{o}C, condition 2 = Prior incubation in fridge (4^{o}C) for 24 hours

4.3 Comparison of Antibacterial Activity of Isolated Endophytic Bacteria and Endophytic Fungi with Commercial Antibiotics

The endophyte's antibacterial activity was compared with that of five commercial antibiotics, whose ZOI were measured based on the disc diffusion method (Table 6). Among the 5 commercial antibiotics, only Enrofloxacin presented with antimicrobial activities against both ATCC *E. coli* and ESBL-EC and showed the highest activity of the ZOI toward all test bacteria, with the highest ZOI (33 mm) against *S. aureus*, followed by *E. coli* (31 mm), MRSA (15 mm), and ESBL-EC (15 mm). MML(A)2, CL(B)1, KKR(A)1, and F1 isolates, on the other hand, have ZOI ranging from 11 mm to 25 mm against both ATCC and ESBL-EC. MML(A)2 has the highest antimicrobial activity against ATCC *E. coli*, with 25 mm of ZOI, while CL(B)1 has the highest ZOI (25 mm) against ESBL-EC. Only a few isolated endophytes show ZOI against ATCC *E. coli*, with MMS(A/F)1 having the highest antimicrobial activities (ZOI = 15mm-20mm), followed by KKR(A)2 (ZOI = 18mm) and CL(B)2 (18mm). Surprisingly, F2

showed the highest ZOI (35 mm) toward ESBL-EC only in condition 1, which is higher than the ZOI created by enrofloxacin.

On the contrary, all of the selected 5 commercial antibiotics showed ZOI toward *S. aureus*, with enrofloxacin showing the highest activity (33 mm), followed by trimethoprim (23 mm), amoxicillin (20 mm), cephalexin (15 mm), and ampicillin (14 mm). KKL (B)1 has the highest ZOI, which is 40mm and 45mm under condition 1 and condition 2 respectively, showing greater antimicrobial activity than the enrofloxacin while CR(A) 1, MML (A)2, and KKL (B)2 have similar ZOI to enrofloxacin with a range of 30-35mm. KKR (C) 1 and F1 show the same ZOI with trimethoprim, while F2 shows a similar result with cephalexin with the 16 mm ZOI.

The commercial antibiotic that shows highest ZOI activity against MRSA is trimethroprim (30mm), followed by enrofloxacin (15mm) with ampicillin showing the lowest ZOI activity (10mm). CL (B)1, CL(B)2, MML(A)2, KKR (A)1, KKR(A)2 and F0 have greater antimicrobial activity compared to the trimethroprim with the ZOI ranging from 50 mm - 25 mm. Although both F1 and F2 have lower antimicrobial activity (25mm) compared to the endophyte stated above, they have better ZOI result (25mm) compared to other commercial antibiotics except for trimethroprim.

Table 6: ZOI (mm) of commercial antibiotic and isolated endophytes against selected test pathogens

Antibiotics	Zone of inhibition (mm)			
	S. aureus	**MRSA**	***E. coli***	**ESBL-EC**
AMP 25	14	-	-	-
AML 10	20	12	-	-
CL 30	15	-	-	-
W 5	23	30	-	-
ENR 5	33	15	31	15
MML (A) 2	35	40*-45	25*	15*
MMS (A/F) 1	-	-	15-20*	-
MMS (B) 2	30	-	-	-
KKL (B) 1	40-45	-	-	-
KKL (B) 2	30	-	-	-
KKR (A)1	-	35-50*	18	15*
KKR (A)2	-	40-50*	18	-
KKR (C) 1	23^	-	-	-
CR(A) 1	30^	-	-	-
CL(A) 1	22-30*	-	-	-
CL (A) 2	20-30*	-	-	-
CL (B) 1	-	35-45*	18^	25*
CL (B) 2	-	30-45*	18^	-
F0	-	25-35*	-	-
F1	23*	25*	11-20*	15*
F2	16	20*	-	35

-: no zone of inhibition observed

*****: ZOI shown after incubation under condition 2

^: ZOI shown after incubation under both condition 1 and condition 2

4.4 Molecular identification of the Endophyte

Ten isolated endophytic bacteria (CL(B)1, KKR (C)1, CR(A)1, MMS(B)2, KKR(A)1, CL(B)2, KKL(B)1, KKR(A)2, CL(A)1, MML(A)2) and 3 endophytic fungi (F0, F1, F2) with antimicrobial properties were selected and further determined by using molecular technique. The universal fungal primers ITS1/ITS4 and EF1-1018F/EF1-

1620 R primers were used for endophytic fungi identification, while Taf/Tar primers were used for endophytic bacteria identification.

In this study, the amplification of the highly conserved ITS region and the EF1 region showed negative results for Fungi 0, Fungi 1, and Fungi 2 (Figure 21). However, the amplification of the highly conserved Tet region showed a positive result for Bacteria 1, 2, 4, 5, 6, 7, 8, and 9, although Bacteria 1 and 4 only showed a light band during the first Polymerase Chain Reaction (PCR) (Figure 22). The PCR was repeated with a higher volume of bacterial DNA (5 ul to 10 ul), and the annealing temperature was reduced from 62 ^{o}C to 61 ^{o}C. A positive and clearer band was obtained for bacteria 1, 2, 3, 7, and 10 (Figure 23). The size of amplified PCR products is approximately 450 bp. BLAST result using primer 16s rRNA showed that bacteria 1, 5, 6, 8, and 10 has 100% of sequence similarity with *Calidifontibacillus erzurumensis (*also share 100 % similar sequences with *B. velezensis, B. atrophaeus, B.vallismortis),* sample 2 shows 100% of similar sequence with *Streptococcus caprae* (also share similar sequence with *S.epidermitis, S. capitis)* ,sample 3 and 9 shows 100% of similar sequence with *Bacillus clarus (*share 100% similarity with *B.wiedmannii, B.tropicus, B. proteolyticus)* while sample 7 shows 100% similar sequence with *Lysinibacillus boronitolerans* (also share similar sequence with *L. macroides, L. pakistanensis, L. xylanilyticus)* (Figure 26, 27, 28, 29) .

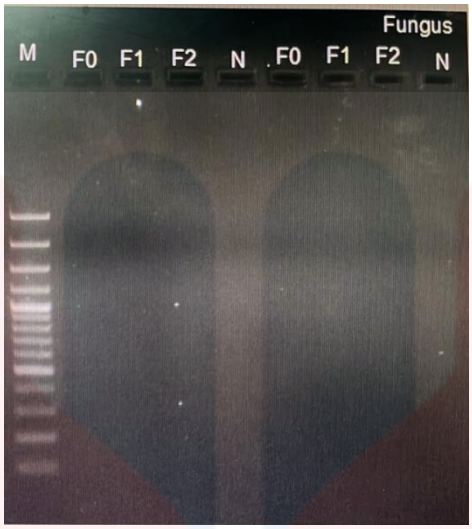

Figure 21: The amplification of ITS and EF1 regions showed a negative result.
Keys: M- 100 bp Plus DNA Ladder as Marker; N- negative control; F0, F1, F2- culture from SDA with ITS and EFI primers where F0 = Fungus 0, F1= Fungus 1, and F2 = Fungus 2

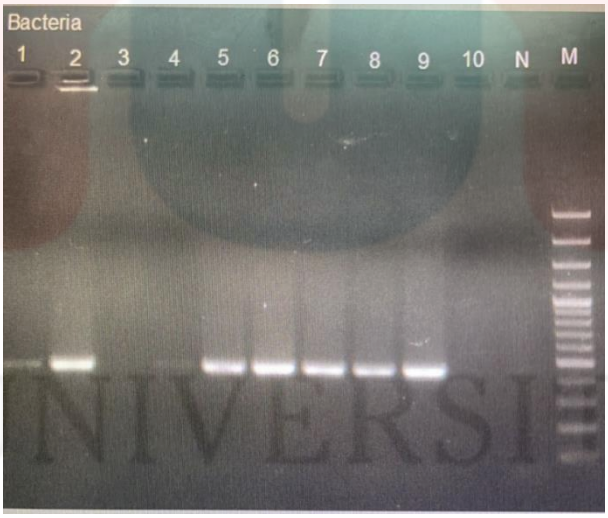

Figure 22: The amplification of *Tet* region showed a positive result for bacteria 1, 2, 4, 5, 6, 7, 8, and 9.
Keys: M- 100 bp Plus DNA Ladder as Marker; N- negative control; 1,2,3,4,5,6,7,8,9,10 are bacterial culture from Nutrient agar with Tar and Taf primers where 1 = CL(B)1, 2 = KKR(C)1, 3 = CR(A)1, 4 = MMS(B)2, 5 = KKR(A)1, 6 = CL(B)2, 7 = KKL(B)1, 8 = KKR(A)2, 9 = CL(A)1 and 10 = MML(A)2

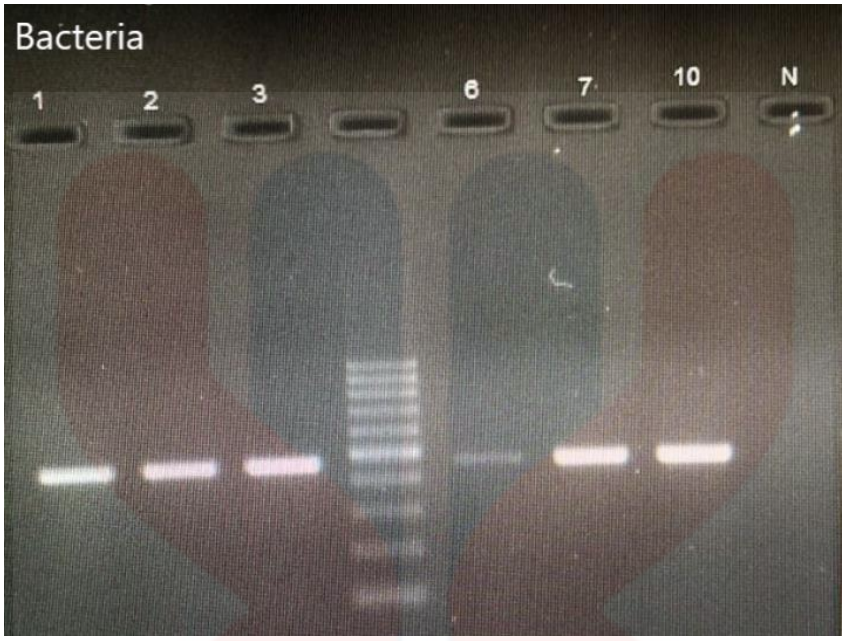

Figure 23: Strong positive band was obtained after increasing the volume of bacterial DNA to 10 ul and changing the annealing temperature to 61^{o}C.
Keys: M- 100 bp Plus DNA Ladder as Marker; N- negative control; 1, 2, 3, 6, 7, 10 are bacterial culture from Nutrient agar with Tar and Taf primers where 1 = CL(B)1, 2 = KKR (C)1, 3 = CR(A)1, 6 = CL(B)2, 7 = KKL(B)1, and 10 = MML(A)2

5.0 DISCUSSION

Endophytes are microorganisms that are widely distributed in plants, which account for 82% of all life on earth. Endophytes appear as a different source of bioactive natural products with potential applications in the pharmaceutical industry because of their enormous diversity and untapped potential. Consequently, the pharmaceutical industry has become interested in endophytes as it has most significant and promising source of new antibiotics for use against susceptible and resistant types of microorganisms. The development of antibiotic resistance in microbes, particularly pathogens, makes the search for new and innovative antibiotics an ongoing process. Two of the typical resistant strains linked to hospital-acquired infections are Methicillin-Resistance *Staphylococcus aureus* and Extended-Spectrum Beta-Lactamase-producing *Escherichia coli* (ESBL-EC) but only few therapeutic options are currently available to manage MRSA and ESBL-EC infections (Dancer *et al.*, 2003). In this study, some of the endophytes isolated from the chosen plant samples showed potential antimicrobial activity against these resistant varieties. Future research on the bioactive compound using such an endophyte may therefore offer more diverse therapeutic options.

Interestingly, despite the use of Sabouraud Dextrose Agar (SDA) in this study, the majority of the endophytes that were isolated were bacteria, and only four endophytic fungi were isolated from the plant samples. The use of media and incubation conditions that are more conducive to the growth of the obtained organism may be the cause. According to a study on the effects of various culture media on isolating endophytic fungi conducted by Verma *et al.* (2011), Mycological agar (MCA) is the best agar to isolate endophytic fungi, showing the highest number of isolates (95 isolates) with the greatest species richness. MCA consists of 10 g papain digest of soybean meal, 10 g

dextrose, 15 g agar, 0.4 g cycloheximide, and 50 mg chloramphenicol, which might favour the growth of fungi and suppress the growth of bacteria due to the presence of antibiotics (Verma *et al*., 2011). However, SDA agar, which contains 10 g peptones, 20 g agar, and 40 g dextrose with a pH of 5.6, not only allows the growth of fungi but also some other bacteria, such as *Bacillus* spp., due to the ability of its spores to utilise dextrose sugar for metabolism.

For the fungal sample in this study, two sets of universal primer for fungal, ITS and EF1, were used. Using both ITS and EF1 primers, however, did not produce any results. According to Peay *et al*. (2008) and Raja *et al.* (2017), the most frequently sequenced DNA regions in fungi are the ITS and EF1 regions. The inability to generate Polymerase Chain Reaction (PCR) products from fungal isolates may be attributable to the fungus' low DNA concentration. According to Caister Academic Press, the amount of total DNA in a PCR has a significant impact on the procedure's outcome. When the ratio of target DNA to burden DNA is extremely low and the total amount of DNA in a PCR reaction is extremely small, the probability of its loss to any conceivable cause such as clotting, adsorption, chemical or enzymatic degradation will increase. In addition, a small quantity of target DNA also increases the likelihood of DNA contamination by impurities on anything that can encounter with the DNA solution. In this study, the low DNA concentration was obtained due to a lack of proper fungal DNA extraction kits and enzymes such as lyticase and zymolase, which are essential for breaking open the cell walls of fungi and yeast, which may be the cause of the low DNA concentration in fungi. The primary component of zymolyase is beta-1,3-glucan laminaripentaohydrolase, which hydrolyzes glucose polymers connected by beta-1,3-bonds to generate laminaripentaose (Fuxman Bass *et al.*, 2016).

In this study, the annealing temperatures for the first PCR and the second PCR were set at 62^{o}C and 61^{o}C, respectively. Comparing the results of both PCRs with different annealing temperatures, the results of the second PCR are more satisfying, as no smeared bands were observed. According to Zrimec *et al*. (2013), band smearing could be caused by imperfectly paired strands of amplified DNA, and in this case, an overly high annealing temperature could be to blame. A high annealing temperature prevents optimal primer-template binding, whereas a low annealing temperature can result in the formation of non-specific duplexes or intramolecular hairpins, both of which reduce reaction efficiency. According to the results, the optimal annealing temperature for 16S rRNA is 61^{o}C.

In this study, the bacteria were identified by using 16S rRNA sequencing. The ubiquity and evolutionary properties of the 16S rRNA gene make it an important molecular marker. 16S rRNA sequencing is widely used for the identification of bacteria and phylogenetic studies due to the fact that the 16S rRNA gene is present in nearly all bacteria and frequently exists as a multigene family, or operon. Second, the function of the 16S rRNA gene has not changed over time, proposing that random sequence changes are a more accurate measure of time, and the 16S rRNA gene consists of 1,500 base pairs which is sufficiently large for informatics purposes (Janda *et al*., 2007). Based on the BLAST result utilising primer 16S rRNA, it was determined that bacteria 1, 5, 6, 8, and 10 have similar sequences to *Calidifontibacillus erzurumensis, Bacillus. velezensis, B. atrophaeus,* and *B. vallismortis.* Sample 2 shares 100% sequence similarity with *Staphylococcus. caprae, S. epidermitis*, and *S. capitis*, whereas samples 3 and 9 share 100% sequence similarity with *Bacillus. clarus, B. wiedmannii, B. tropicus,* and *B. proteolyticus.* The sequence of Sample 7 is identical to that of

Lysinibacillus boronitolerans, L. macroides, L. pakistanensis, and *L. xylanolyticus.* Despite the fact that 16S rRNA gene sequencing is extremely useful for bacterial classification, it has low phylogenetic power at the species level and poor discriminatory power for some genera, and DNA relatedness studies are required to provide absolute resolution of these taxonomic issues (Janda *et al*., 2007). Bacillus is an excellent example of this. On the basis of their 16S rRNA genes, the type strains of *B. globisporus* and *B. psychrophilus* share >99.5% sequence similarity. However, at the DNA level, they exhibit only 23 to 50% relatedness in reciprocal hybridization reactions. In addition, a number of studies have demonstrated 16S rRNA gene sequence similarities or identities between the *Streptococcus mitis* group and other non-fermenters. In such situations, 16S rRNA gene sequence data cannot provide a definitive answer because they are unable to differentiate between recently diverged species. To address this issue, Drancourt *et al.* (2000) have proposed several criteria for 16S rRNA gene sequencing as a reference method for bacterial identification, including the inclusion of full 16S rRNA gene sequences whenever possible, and the use of microarray-based technologies in conjunction with 16S or other housekeeping gene targets may provide a much more sensitive and definitive platform for molecular species identification.

According to the findings of this study, endophytes were isolated from water spinach, cassava, and Mexican mint. Surprisingly, *Calidifontibacillus erzurumensis, Staphylococcus caprae, Bacillus clarus, Lysinibacillus boronitolerans,* and a few fungi were isolated from the three previously mentioned plant samples demonstrating antimicrobial activity against ATCC *E. coli, S. aureus,* ESBL-EC, and MRSA. Using the agar plug diffusion method, the results show that the bacteria

were able to inhibit the growth of the test pathogens. *C. erzurumensis* has the highest Zone of inhibition (ZOI) against ATCC *E. coli* and ESBL-EC under condition 2, which is 24 hours of refrigeration at 4˚C followed by incubation at 37˚C. Since the metabolites produced by bacteria can be affected by environmental factors such as temperature (Horak *et al.*, 2019), this could suggest that low temperatures favour the production and diffusion of *C. erzurumensis*-produced metabolite. For *S. aureus, C. erzurumensis* and *B. clarus* showed the highest antimicrobial activity with larger ZOI under condition 2 which highly suggest that cool environment favour these bacteria to produce bioactive compound. For MRSA, *C. erzurumensis* showed the greatest antimicrobial activity under condition 2 as well followed by fungus 0, fungus 1 and fungus 2. Generally, more isolated endophytes showed antimicrobial activity and a larger ZOI toward *S. aureus* and MRSA compared to ATCC *E. coli* and ESBL-EC. According to Breijyeh *et al.* (2020), Gram-positive bacteria are more susceptible to antimicrobial compound compared to Gram-negative bacteria. This is because of the outer membrane structure of Gram-negative bacteria. To reach their targets, the majority of antibiotics must pass through the outer membrane; for instance, hydrophobic drugs can pass through a diffusion pathway; hydrophilic antibiotics, such as β-lactams, pass through porins; and vancomycin, due to its structure, cannot cross the outer membrane and therefore cannot use any of these passages. Any minor alteration of the outer membrane of Gram-negative bacteria, such as a change in hydrophobic properties or mutations in porins, can result in resistance. Since Gram-positive bacteria lack of this important layer, which make them more susceptible to antimicrobial drug (Breijyeh *et al*., 2020).

One of the endophytic bacteria isolated, *Calidifontibacillus erzurumensis* which was isolated from all the plant samples showed encouraging antibacterial activities against

the test pathogen. With the presence of bioactive compounds that are able to inhibit the growth of a test pathogen, further study of the mechanism of the bioactive compound can be done to develop a better understanding. Until now, there has not been much study done on these bacteria, as it was first isolated from a hot spring in Turkey in December 2020 and was categorized as a novel Gram-positive bacterium (Adiguzel *et al*., 2020).

The isolation of *S. caprae* from the water spinach was an unexpected discovery, as this bacterium had previously been isolated from goats and was also commensal on human skin. However, it shows antimicrobial activity toward *S. aureus.* According to Uddin *et al.* (2021), *S. hominis* strain MBL_AB63, another species of Staphylococcus that is also commensal on human and animal skin, was isolated from a jute seed and showed promising antimicrobial activity against *S. aureus* SG511 when screening for antimicrobial substances. In another study, *S. warneri*, also one of the commensal bacteria on human skin was isolated from fruit. Although the occurrence of *Staphyloccocus spp*. is unusual, it might be due to the changing ecology of bacteria, which has adapted to the inner tissue of plants (Phukon *et al*., 2013).

Bacillus clarus, a cassava isolate, was previously classified as *B. cereus*. However, it was proposed as a new species known as *B. clarus* based on the biochemical and informatics characterization of this bacteria with strain ATCC 21929 (Mendez *et al*., 2019). Although there is no report supporting that *B. clarus* has antibacterial properties, there is one article reporting that *B. clarus* is effective against *Fusarium graminearum* and showing more than 70% growth inhibition of fungal mycelium, and it has been proven that the disease index of wheat head scab caused by *F. graminearum* has decreased by around 80% by managing the disease with *B. clarus* NOK09, *B. subtilis*

NOK33, and *B. amyloliquefaciens* NOK109 (Kaul *et al.*, 2022). In this study, based on the agar plug diffusion method, this microorganism shows antimicrobial properties toward *S. aureus*. However, there is no significant difference in terms of the ZOI between condition one and condition two. This might indicate that the production of bioactive compounds by these bacteria is not influenced by the temperature.

Lysinibacillus boronitolerans was previously described as *B. sphaericus*, was isolated from water spinach. *L. boronitolerans* is a diazotrophic endophyte that has been demonstrated to be effective against *Rhizoctonia solani*, the fungus that causes rice sheath blight (Shabanamol *et al.*, 2017). Based on the research by Ahmad *et al*. (2014), 125 strains of Lactobacillus were isolated from fruit and tested against bacterial and fungal foodborne pathogens. An isolated *Lysinibacillus* spp. exhibited significant aggregation-co-aggregation probiotics properties and inhibits the foodborne Gram-positive microbial pathogens such as *Staphylococcus aureus, Staphylococcus epidermidis* and *Bacillus cereus*. In this study, *L. boronitolerans* demonstrates antimicrobial activities against S. aureus with 40-45mm of ZOI. The ZOI under condition 2 is 45mm, which is greater than the ZOI under condition 1, indicating that cooler temperatures promote the production of antimicrobial bioactive compounds. The ability to produce bioactive compounds with antimicrobial properties demonstrates that these microorganisms may serve as models for the development of new drugs and highlights the significance of studying endophytes as a new source of medication.

6.0 CONCLUSION

Endophytes represent a group of microorganisms worth investigating as they are rich in biometabolites and may serve as reliable sources of compounds with high potential antimicrobial properties. In this study, three endophytic fungi and ten endophytic bacteria with antimicrobial properties were isolated, and sequencing was done to identify the endophytic bacteria. The isolated endophytic bacteria have 100% sequence similarity with *Calidifontibacillus erzurumensis* (which also shares similar sequence with *Bacillus velezensis, B. atrophaeus, and B. vallismortis*), *Staphylococcus caprae* (which also shares similar sequence with *S. epidermitis and S. capitis*), *Bacillus clarus* (which also shares similar sequence with *B. wiedmannii*), and *Lysinibacillus boronitolerans (share similar sequence with L. macroides, L. pakistanensis,* and *L. xylanolyticus).* These bacteria have shown antimicrobial activities against the tested antibiotic resistant pathogens namely, Extended-Spectrum Beta-Lactamase-*Escherichia coli* (ESBL-EC) and Methicillin-Resistance *Staphylococcus aureus* (MRSA). However; further research on the antimicrobial compound produced by these bacteria needs to be done to generate a better understanding of the mechanism of their antimicrobial activities, which may help in developing new and effective antimicrobial drugs. Furthermore, due to the limitations of 16S rRNA sequencing, a complete genome sequencing is required to identify the exact species of bacteria.

7.0 RECOMMENDATIONS

1. Prior to performing agar plug diffusion assays, molecular identification should be performed to confirm the reliability of morphological identification.
2. For more reliable and enhanced isolation and identification of endophytic fungi, antibiotics should be added to Sabouraud Dextrose Agar (SDA) to suppress the growth of bacteria. The antibiotics that can be used to inhibit bacterial growth and most contaminant fungi are chloramphenicol and cycloheximide, respectively.
3. Complete genome sequencing needs to be carried out to identify the species of endophyte accurately.
4. A proper fungal DNA extraction kit should be used to extract fungal DNA.
5. More research should be done to learn more about the metabolites that the endophyte makes that have antimicrobial properties.
6. Thin Layer Chromatography (TLC) method can be used for further analysis of the presence of active compounds, metabolites, and phytochemicals from the endophytes.
7. The isolated endophyte that showed antibacterial activity in primary screening should be subjected to aerobic liquid fermentation and tested for its potential against test pathogens by the disc diffusion method. This is because the selected endophyte that showed antibacterial activity against one of all test pathogens in primary screening might not show significant antibacterial activity when subjected to secondary screening by using ethyl acetate extract. This observation was also reported in several studies in which endophytic fungi showed activity in solid media but not when subjected to fermentation. This was

attributed to the possible contribution of the type of media and cultural conditions in the biosynthesis of the active metabolites (Marcellano *et al.*, 2017).

8. All the ethyl acetate extracts of isolated endophytes with antimicrobial activities need to be tested for MIC to see if they are showing significant antibacterial activity against the test pathogen. In this way, the crude extracts would have generated more potent compounds that have greater antimicrobial activity once purification is done (Fabry *et al.*, 1998; Devi *et al.*, 2012).
9. Further research into the antimicrobial, biocompatibility, and toxicity of the active compounds is warranted and required to validate the findings.

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APPENDICES

APPENDIX A: PICTURES OF AGAR PLUG DIFFUSION ASSAYS ON SELECTED TEST PATHOGENS

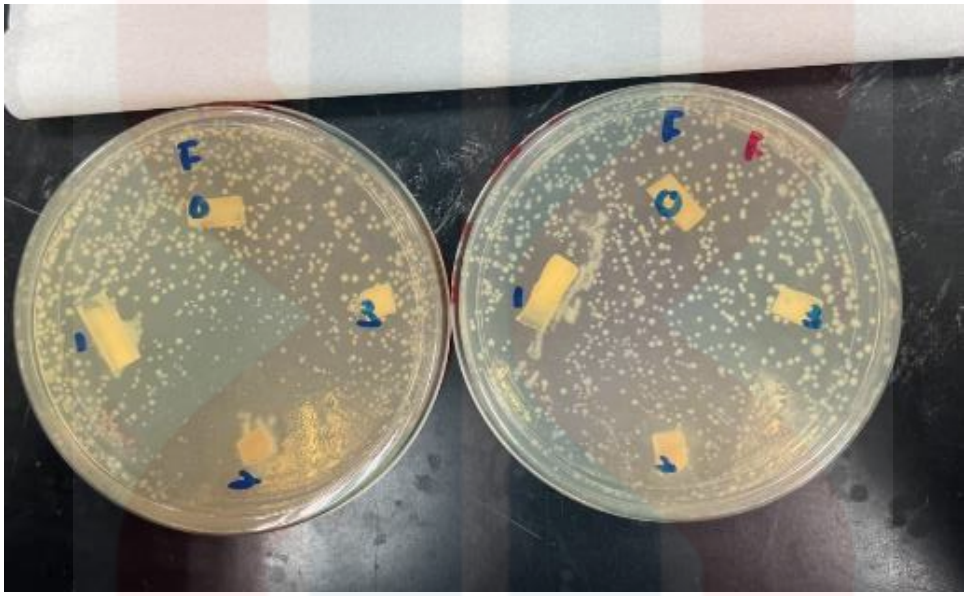

Figure 24: The isolated fungi show ZOI on ESBL-producing *E. coli* under two different conditions. (Left plate was incubated under condition 1 while right plate was incubated under condition 2)

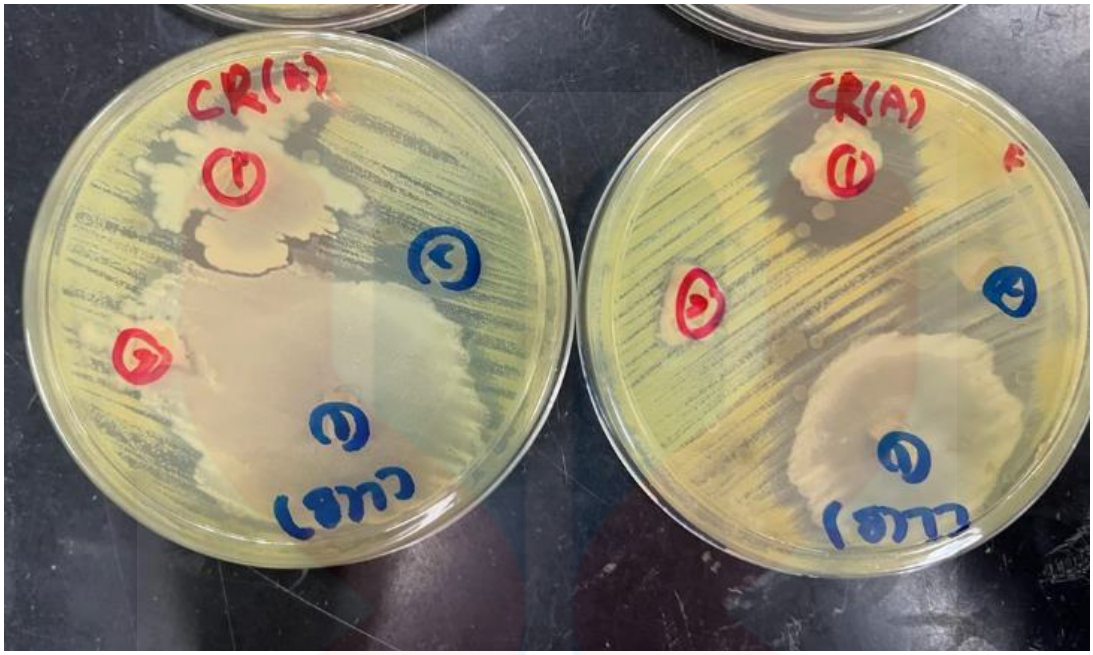

Figure 25: The isolated bacteria show ZOI on *S.aureus* under two different condition. (Left plate was incubated under condition 1 while right plate was incubated under condition 2)

APPENDIX B: PICTURE OF SEQUENCING RESULT

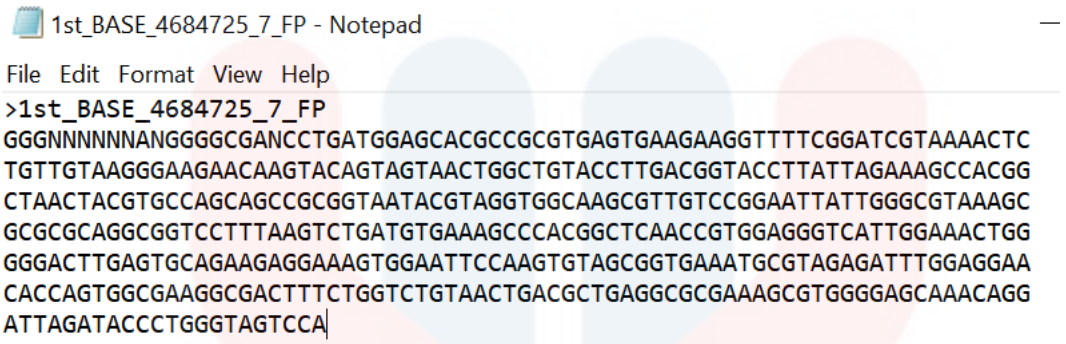

Figure 26: Sequencing result of sample 7 (KKL(B)1) with forward primer

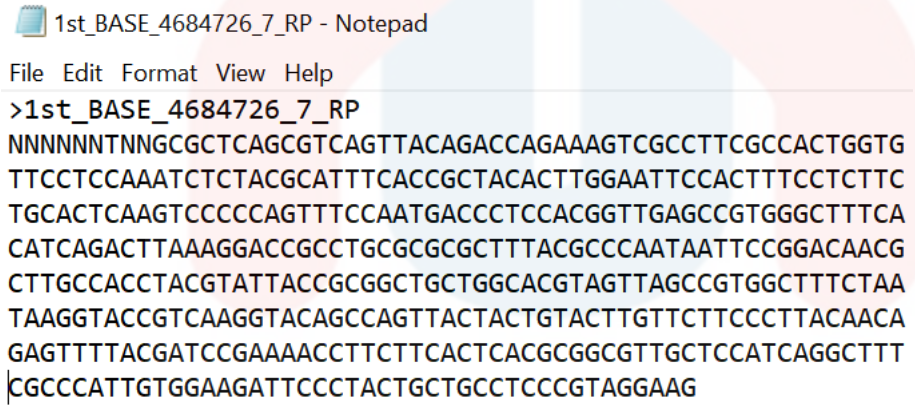

Figure 27: Sequencing result of sample 7 (KKL(B)1) with reverse primer

Figure 28: Sequence Alignment result of sample 7 (KKL (B) 1)

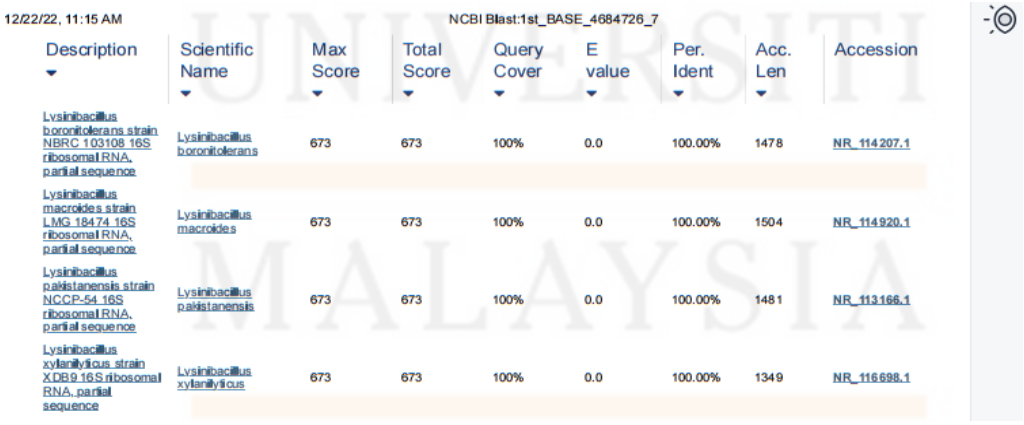

Figure 29: BLAST result of sample 7 using the 16s rRNA primer showed 100% of sequence similarity with *Lysinibacillus boronitolerans, L. macroides, L. pakistanensis*, and *L. xylanolyticus*