Assessing Antibiotic Resistance in the Skin Microbiome of Elasmobranchs: A Comparative Analysis

By

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ABSTRACT

Antimicrobial resistance is a growing global health concern, and a One Health Perspective is being used to tackle the problem. Anthropogenic pollutants, such as antibiotics from runoff, contribute to and accelerate the spread of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) in marine ecosystems. Elasmobranchs, as top predators in marine ecosystems, may be good bioindicators for detecting environmental contamination. My research investigates the diversity and antibiotic resistance patterns of skin microbiomes in six elasmobranch species (sharks, rays, and skates) from the Gulf St. Vincent in Australia, focusing on their role in marine antimicrobial resistance (AMR) in the marine environment. Their skin microbiomes, composed of bacteria such as *Pseudoalteromonas* and *Psychrobacter*, were examined to assess their antibiotic resistance patterns and the presence of ARGs. The study involved:

- Culturing microbes from elasmobranchs' skin.
- Performing antimicrobial susceptibility testing on eight common antibiotics.
- Sequencing microbial genomes to identify ARGs.

Results revealed that *Pseudoalteromonas* and *Psychrobacter* were predominant across most hosts, indicating their potential adaptation to the skin environment. Significant resistance to Penicillin was observed in 84 % of the microbes tested, while there were low resistance rates for Sulphafurazole, Chloramphenicol, Tetracycline, and Erythromycin. Whole genome sequencing of selected microbes via the MinION device revealed several ARGs, including those for efflux pumps and beta-lactamases. However, inconsistencies existed between 'Specialty Genes' ARGs identified by the BV-BRC annotation platform (2024) and the resistance identified from antimicrobial susceptibility tests. These inconsistencies highlight the complexity of resistance mechanisms and the need to integrate genomic and phenotypic data.

In conclusion, my study highlights the diversity of microbes and antibiotic resistance patterns in the skin microbiome of six elasmobranch species, with *Pseudoalteromonas* and *Psychrobacter* being predominant. Apart from resistance to Penicillin, most

elasmobranch microbes were sensitive to the antibiotics, suggesting a limited flow of ARGs into the shark microbiome. Therefore, sharks are not currently a mechanism of moving antibiotics in the marine environment in these locations.

Only eight cultured microbes were fully sequenced, while the majority relied on 16S rRNA sequencing, limiting a comprehensive understanding of microbial genomes. Inconsistencies between potential antibiotic resistance genes identified by BV-BRC analysis and antimicrobial susceptibility test results underscore the complexity of resistance mechanisms. Further research, including complete genome sequencing of more microbial samples, is essential to improve the understanding of the antibiotic resistance genes and microbial adaptation in these marine organisms. The ecological importance of elasmobranchs and their role in maintaining marine biodiversity, as well as understanding their skin microbiomes and associated resistance patterns, provides a valuable perspective on the impact of AMR in marine ecosystems.

DECLARATION

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed  ……

Name: Sein Hwang

Date…….31/10/2024

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Chapter One: Introduction

1. Introduction

Antimicrobial resistance (AMR) poses significant challenges to global health under the One Health Perspective, which recognizes the interconnectedness of human, animal, and environmental health systems (Vasala et al., 2020). Antibiotic-resistant bacteria (ARB) develop through genetic mutations and horizontal gene transfer (HGT), facilitating the spread of resistance genes between bacterial populations (Munita & Arias, 2016). Human activities, including industrial pollution, agricultural runoff, and excessive antibiotic use, have accelerated the spread of AMR, particularly in aquatic environments (Okoye et al., 2022). The marine ecosystem, heavily impacted by these pollutants, serves as a reservoir for antibiotic resistance genes (ARGs) and ARB (Nogales et al., 2011). In marine organisms, such as fish and elasmobranchs (sharks, rays, skates), the skin microbiome plays a critical role in immune defence and provides insights into environmental contamination (Doane et al., 2017; Goodman et al., 2024; Kerr et al., 2023). Testing for AMR involves methods like antimicrobial susceptibility testing and whole-genome sequencing, which, coupled with bioinformatics tools, allow for comprehensive analysis and tracking of resistance patterns (Dinsdale et al., 2008; Johri et al., 2019) and the spread of ARG into the environment.

1.1 Background of Antimicrobial Resistance (AMR) and Antibiotic-Resistant Bacteria (ARB)

Antimicrobial resistance (AMR) defines the ability of microorganisms, such as bacteria, viruses, fungi, and parasites, to resist the effects of antibiotic drugs that were once effective against them (Aslam et al., 2018). AMR is widely recognized in human diseases, but from a One Health perspective, it also presents significant global health concerns in animal and environmental sustainability (Velazquez-Meza et al., 2022).

In AMR development in bacteria, when bacteria evolve to resist antimicrobial drugs it results in treatment becoming ineffective (Baquero et al., 2021). The evolution of

antibiotic resistance bacteria (ARB) occurs through genetic mutation and/or the spread of antibiotic resistance genes (ARGs) via horizontal gene transfer (HGT) (Munita & Arias, 2016). HGT spreads ARGs between bacteria through conjugation (involving direct DNA transfer), transformation (uptake of environmental DNA), and transduction (transfer by bacteriophage) (Jian et al., 2021).

Antibiotic-resistant bacteria (ARB) naturally occur in various environments, including water, soil, air, and biological hosts. However, human activities, particularly pollution from industrial, agricultural, and domestic sources, are introducing new resistance factors into the environment. These anthropogenic influences accelerate the spread of AMR in ways that are difficult to predict (Larsson & Flach, 2022). Contaminants such as antibiotics move through water systems, spreading from land to the ocean and back again, creating a cycle that affects humans, animals, and entire ecosystems. These continuous exchanges between terrestrial and marine environments increase the prevalence of AMR, posing a critical threat to environmental health and biodiversity (Wang et al., 2024).

1.2 AMR in the marine environment

Pollutants in marine environments such as antibiotics and ARBs by Emerging Contamination (EC) such as pharmaceutical chemicals from industrial and hospital wastewater, and excess antibiotic use in agriculture enter marine environments, increasing ARGs and, driving the selection and spread of ARB in the marine environment (Okoye et al., 2022). From 2012 to 2023, antibiotics have been found in high concentrations near anthropogenic activity areas (e.g., hospitals, industrial effluents, and livestock farms) where they can enter water systems through direct discharges or runoff from fields, contributing to the spread of ARGs (Bernier & Surette, 2013; Brauge et al., 2024; Gross et al., 2022).

Terrestrial pollutants caused by anthropogenic activity affect the health, reproduction, and associated microbial communities of marine organisms (Nogales et al., 2011). The marine organism microbiomes on their skin play an important role in the immune defence

system, contributing to wound healing, and protecting against the invasion of foreign material invasion on their skin such as toxic chemicals, and toxic heavy metals (Doane et al., 2017). While gut microbiomes are often investigated in terrestrial animals and humans, for marine organisms the skin is preferred because it is early accessible and does not require the expiration of the animal. In addition, faecal material is difficult to retrieve in the marine environment, i.e. it is lost to the water column (Ross et al., 2019; Sehnal et al., 2021)

1.3 Microbiome as indicators for measuring marine pollution

A microbial biofilm is a protective community of microorganisms attached to surfaces, embedded in a self-produced matrix, which enhances their survival and resistance in various environments (Grumezescu, 2017). In the marine environment, microbial taxa from within the host biofilm can be investigated to provide a measure of health of host. The microbiome of host species such as marine mammals, fish, coral, and shellfish has been shown to with host health (Di Natale et al., 2023; Kelly et al., 2014; Robles-Malagamba et al., 2020; Wei et al., 2024).

The microbiome serves as a crucial biomarker for detecting environmental changes and contamination (Callewaert et al., 2020; Raina et al., 2010). The spread of antibiotic-resistant microbes into marine environments can be identified by testing the water column or the organisms inhabiting these locations (Pepi & Focardi, 2021). Fish, in particular, are valuable indicators of environmental contamination; ARGs can be detected by sampling their skin (Miranda et al., 2024). Exposure to high levels of antibiotics in fish correlates with a higher relative abundance of ARGs. This increased antibiotic exposure accelerates the development of resistance in non-resistant bacteria, ultimately raising the overall resistance levels within the microbiome (Hossain et al., 2022).

Elasmobranchs (sharks, rays, and skates) are a vital group of fish, functioning as top predators in the food chain and playing a key role in maintaining ecological balance (Afonso et al., 2022). They are also excellent bioindicators of pollution, as biomarker analyses of their microbiomes—found in their skin, organs, gills, and blood—can provide insights into environmental contamination (Alves et al., 2022; Perry et al., 2021). The

skin microbiome of elasmobranchs is particularly significant, as it helps protect against pathogens, supports wound healing, and contributes to their overall immune defence. This microbiome is influenced by the structure of the dermal denticles on their skin, which aid in controlling microbial attachment and biofilm formation (Goodman et al., 2024).

1.4 Common Skin-Associated Bacterial Species on Elasmobranchs and Their Microbiome Characteristics

Despite their ecological importance, microbial communities on elasmobranchs have been studied in only a limited number of species (Alves et al., 2022). The microbiomes of sharks commonly include members of the Pseudomonadota phylum, particularly families such as *Pseudoalteromonadaceae, Pseudomonadaceae, Vibrionaceae*, and *Alteromonadaceae* (Doane et al., 2017; Doane et al., 2020) while rays host novel species from *Rhodobacteraceae, Moraxellaceae, Caulobacteraceae, Alcanivoracaceae*, and *Gammaproteobacteriathe,* are members of the microbiome (Doane et al., 2017; Doane et al., 2020; Kerr et al., 2023). Sharks tend to have consistent microbiomes due to their densely packed dermal denticles, whereas rays, with sparser denticle arrangements and thick mucus layers, exhibit more diverse microbial communities (Kerr et al., 2023). Skates, specifically during embryonic development, host microbial communities dominated by Pseudomonadota, Bacteroidota, and Planctomycetota (Mika et al., 2021), and there was a dynamic shift in microbial colonization during different stages of development (Mika et al., 2021).

Geographic factors, water pH, salinity, and proximity to pollutants shape these microbiomes. Sharks collected from wild and captive environments showed no significant changes in diversity but displayed variations in microbial abundance (Goodman et al., 2022). Dermal denticle morphology and host health also influence microbial composition and abundance (Pogoreutz et al., 2019).

1.5 Movement and Mechanism of AMR

In humans, AMR is driven by misuse of antibiotics, incomplete treatment regimens, and excessive antibiotic use in agriculture and livestock production (Llor & Bjerrum, 2014). Similarly, in marine environments, natural microbial interactions in elasmobranch mucus layers can contribute to AMR. For example, these microbes may naturally produce antibiotics to protect their hosts, creating selective pressures that promote ARG development (Bengtsson & Greko, 2014; Manyi-Loh et al., 2018).

In the marine environment, AMR is not only driven by anthropogenic pollutants. Recent studies have shown that microbes residing in the mucus layers of elasmobranchs, such as rays and skates, naturally produce antibiotics that inhibit pathogenic bacteria, protecting their hosts (Ritchie et al., 2017). However, this natural antibiotic production also applies selective pressure on the microbial environment, encouraging the evolution of resistance in other bacteria, leading to the emergence and spread of ARGs (Muteeb et al., 2023; Ritchie et al., 2017). These ARGs contribute to the complex dynamics of AMR in marine environments (Nappier et al., 2020).

Natural selection plays a significant role, as bacteria and other microorganisms naturally evolve through genetic mutations and the spread of antibiotic resistance genes (ARGs) (Vassallo et al., 2021). These genes are exchanged between different bacteria via mobile genetic elements (MGEs), including plasmids, transposons, and integrons (Khedkar et al., 2022). ARGs confer resistance to multiple antibiotics (Table 1). The mechanisms of resistance include reducing intracellular antibiotic concentrations through efflux pumps, inactivating antibiotics via hydrolysis or modification, altering or protecting antibiotic targets, and decreasing cellular permeability to antibiotics (Huang et al., 2022).

Table 1. Mechanisms of antibiotics: overview of antibiotics in susceptibility testing, including their mechanisms of action, target genes, advantages, and disadvantages

Antibiotic	Mechanism of Action	Advantage	Disadvantage	Target Gene	Reference

Sulfafurazole 300ug (SF300)	Sulfafurazole disrupts the production of folic acid in bacteria by competing with para-aminobenzoic acid (PABA) for access to the active site of the enzyme dihydropteroate synthase.	Effective against a broad range of bacterial infections.	Potential for high mutation rate in target genes, complicating resistance analysis.	folP	(Im et al., 2019)
Chloramphenicol 30ug (C30)	Chloramphenicol blocks protein synthesis by attaching to the 50S ribosomal subunit, hindering the binding of tRNA and stopping the production of proteins.	Broad-spectrum antibiotic with good tissue penetration.	Resistance can involve multiple mechanisms, making it harder to pinpoint specific resistance genes.	rRNA (23S ribosomal RNA)	(Oong & Tadi, 2024)
Tetracycline 30ug (TE30)	Tetracycline prevents bacterial protein synthesis by attaching to the 30S ribosomal subunit.	Effective against a wide range of bacteria.	Efflux pump-mediated resistance can obscure genetic basis of resistance.	tet genes (e.g., tetA, tetB)	(Chopra & Roberts, 2001)
Erythromycin 15ug (E15)	Erythromycin blocks bacterial protein synthesis by binding to the 50S ribosomal subunit.	Effective against Gram-positive bacteria and some Gram-negative bacteria.	Inducible resistance mechanisms can complicate susceptibility testing.	erm genes (e.g., ermB)	(Farzam et al., 2024)
Ampicillin 10ug (AMP10)	Ampicillin blocks bacterial cell wall synthesis by attaching to penicillin-binding proteins.	Broad-spectrum activity against Gram-positive and some Gram-negative bacteria.	Beta-lactamase production can vary, affecting consistency in resistance testing.	pbp genes (e.g., pbp1A, pbp2B)	(Peechakara et al., 2024)

Streptomycin 10ug (S10)	Streptomycin hinders bacterial protein synthesis by binding to the 30S ribosomal subunit.	Effective against aerobic Gram-negative bacteria.	High likelihood of spontaneous resistance mutations.	rpsL	(Waters & Tadi, 2024)
Penicillin (P1 and P0.5)	Penicillin prevents the cross-linking of peptidoglycan in the bacterial cell wall, causing the cell to undergo lysis.	Highly effective against Gram-positive bacteria.	Resistance often involves multiple genes, complicating genetic analysis	pbp genes (e.g., pbp1A, pbp2B)	(Yip & Gerriets, 2024)

1.6 Testing AMRs

Testing AMR involves several methods to assess microbial susceptibility to antimicrobials. Common approaches include antimicrobial susceptibility testing (AST), such as the disk diffusion method, and minimum inhibitory concentration (MIC) testing (Bayot & Bragg, 2024). In the disk diffusion method, antibiotic-impregnated disks are placed on an agar plate with a bacterial suspension, and the zones of inhibition (clear areas) indicate susceptibility (Berger, 1985). MIC testing determines the lowest antibiotic concentration that inhibits bacterial growth (Gajic et al., 2022; Nappier et al., 2020).

To analyse ARGs in AMR, polymerase chain reaction (PCR) and whole-genome sequencing (WGS) are essential tools for detecting specific resistance genes, offering comprehensive insights into microbial genomes (Shelburne et al., 2017). However, PCR contains limited DNA information, whereas Next-Generation Sequencing (NGS), which is combined with advanced computational and bioinformatic tools, provides powerful strategies for rapid identification and characterization of microbial communities (Dinsdale et al., 2008) and ARGs (Haggerty & Dinsdale, 2017). NGS method using the MinION a portable sequencing device from Oxford Nanopore technologies is useful and enables the complete sequencing of bacterial genomes and mitochondrial genomes and

nuclear regions for elasmobranch genomic analysis (Johri et al., 2019), is rapid and can be conducted remotely, thus enabling tracing AMR's into the field.

Analysis ARGs in bioinformatics, Bacterial and Viral Bioinformatics Resource Centre (BV-BRC) is a tool for ARGs analysis in bioinformatics which provides a unified data model, advanced visualization tools, and comprehensive bioinformatics services, allowing for efficient identification, comparison, and analysis of antimicrobial resistance genes across large genomic datasets (Wattam et al., 2024). In global antimicrobial resistance (AMR) pattern analysis and reference, the Global Antimicrobial Resistance and Use Surveillance System (GLASS) is helping to track and respond to emerging resistance trends (Ajulo & Awosile, 2024).

GAPS IN KNOWLEDGE

Despite the ecological significance of elasmobranchs (sharks, rays, and skates), a significant gap is the lack of comprehensive knowledge about microbial communities within elasmobranchs (Correia Costa et al., 2022). The holobionts of skin microbiome in elasmobranchs have been investigated on 12 species out of 1300 species (Doane et al., 2020; Montemagno et al., 2024). The microbiome of elasmobranch has been investigated with 16S amplicon sequencing. However these studies do not provide any information on the presence of ARGs. To explore the gene content associated with elasmobranch microbiomes, metagenomic studies have started to catalogue the microbial species and gene content present in various elasmobranchs (Doane et al., 2017; Doane, Johnson, et al., 2023; Doane et al., 2020; Goodman et al., 2022; Hesse et al., 2022; Kerr et al., 2023; Perry et al., 2021). Elasmobranch microbiomes are novel, only 7% of all sequences could be identified on the thresher shark (Doane et al., 2017) and 2 out of 5 high-quality Metagenome Assembled Genomes (MAGs) were identified as novel microbial species in sting ray (Kerr et al., 2023). While ARGs occur in the microbiome of elasmobranchs, including on whale sharks, and leopard sharks (Doane, Johnson, et al., 2023; Doane, Reed, et al., 2023), the novelty of the microbiomes suggests that antimicrobial resistance genes are underreported.

Bacteria from the epidermal mucus of rays and skates collected in the USA identified 311 out of 1,860 isolates (16.7%) showing antimicrobial activity, including against vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Ritchie et al., 2017). Ritchie et al.'s (2017) study suggests the use of molecular techniques, such as next-generation sequencing (NGS), to establish a comprehensive baseline of bacterial communities associated with various elasmobranch species and their different habitats.

There is a substantial gap in understanding the specific mechanisms by which marine microbes, acquire and transfer antibiotic resistance. Microbes living in association with marine hosts may have more antimicrobials because they are interacting with other

microbes and the host immune system, via the mucus layers. Because elasmobranchs inhabit shallow waters, are attracted to aquaculture facilities, are long-lived, and accumulate heavy metals, they are a suitable model organism to investigate the movement of antimicrobials in the marine environment.

RESEARCH QUESTIONS, OBJECTIVES AND EXPECTED OUTCOMES

Research questions:

1. How does antimicrobial resistance (AMR) vary across different species of coastal elasmobranchs (Sharks, Rays, and Skates)?
2. What novel genomic information, including antibiotic-resistance genes, can be added to the microbial database from elasmobranch-associated microbiomes?

The objectives of my study:

1. Culture microbes from coastal sharks, rays, and skates to describe whether AMR varied between hosts
2. Test the antibiotic susceptibility of cultured microbes to 8 commonly used antibiotics to describe whether AMR varied between microbial genera
3. Sequence the genomes of selected microbes to describe the presence of antibiotic-resistant genes

Expected outcomes:

1. Identify wether variation in AMR between hosts regardless of microbial genera OR;
2. Variation in AMR Across Microbial Genera regardless of host species
3. Identification of variation of Antibiotic-Resistance Genes
4. Contribution to AMR Monitoring in Marine Systems

Chapter Two: Materials and Methods

2.1 Isolation and Cultivation of Elasmobranch Skin Microbiota

The skin microbes were collected from elasmobranchs inhabiting the Gulf St Vincent, South Australia, Australia. The hosts were freely swimming in the ocean, and collected and released in accordance with the animal ethics. These species included Angel Shark (*Squatina australis*), Eagle Ray (*Myliobatis tenuicaudatus*), Fiddler Ray (*Trygonorrhina dumerilii*), Melbourne Skate (*Dentiraja cerva*), Port Jackson Shark (*Heterodontus portusjacksoni*), and Seven-Gills Shark (*Notorynchus cepedianus*). The sampling was conducted on different dates (June 2022, February 2023, and May 2024) using two distinct methods, as outlined by the Dinsdale Laboratory team (Doane, Johnson, et al., 2023; Kerr et al., 2023). The skin microbiome was chosen as it is the largest organ in the body, and the first line of defence against microbial invasion and has a unique microbiome compared to the water column (Doane et al., 2017; Doane et al., 2020; Kerr et al., 2023). To investigate the skin microbiome of elasmobranchs, microbial samples were cultured on a medium composed of 3.33% Marine Broth 2216 and 2% agar, set in standard petri dishes (15mm x 100mm). These cultures were incubated at 20_°C for 24 hours. Post-incubation, the plates were examined under a microscope to identify distinct microbial morphologies. Selected microbes were streaked to isolated a single culture made into glycerol stocks and stored at -80 ℃ for further analysis.

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Figure 1. The map of elasmobranchs skin microbiome sampling location (ArcGIS, 2024)

2.2 DNA Extraction and Purification from Skin Bacteria

The DNA from each colony was extracted for species identification via sequencing of 16S DNA. DNA extraction and purification were performed using the NucleoSpin® Tissue Kit (Takara Bio Inc.), following the protocols specified (KG, 2014). Initially, 3 to 4 isolated bacterial colonies were treated with 180 µL of Buffer T1 and 25 µL of Proteinase K. This mixture was then incubated at 56°C and periodically vortexed over 3 hours to achieve effective pre-lysis of the samples. Following this, 200 µL of Buffer B3 was added and the samples were again vortexed. The samples underwent further incubation at 70°C for 10 minutes to complete the lysis process. For optimal DNA binding conditions, 210 µL of 96–100% ethanol was added to each sample, followed by vigorous vortexing. The entire mixture, including any visible precipitates, was then transferred to a NucleoSpin® Tissue Column. The subsequent steps—binding DNA to the silica membrane, washing, drying, and eluting purified DNA—were carried out by centrifugation at 11,000 RPM, by the manufacturer's guidelines. The final DNA product was eluted in 100 µL and its concentration was quantified using a Qubit 4 Fluorometer (ThermoFisher, MA, USA).

2.3 PCR Product Amplification

For the PCR analysis, LongAmp™ Taq 2X Master Mix (New England Biolabs, MA, USA) was utilized, adhering to the manufacturer's instructions provided by New England Biolabs (2024). For the PCR setup, a total reaction volume of 25 µL per sample was achieved in the following order. At first, 11.5 µL of nuclease-free water was pipetted into the PCR tube. This was followed by the addition of 12.5 µL of LongAmp™ Taq 2X Master Mix (New England Biolabs, MA, USA). Subsequently, 0.5 µL each of the 10 µM 16s rRNA forward and reverse primers were introduced. Finally, 1 µL of DNA, containing less than 1000 ng, was added to complete the mixture. The thermocycling conditions for a routine PCR performed using the Mastercycler® nexus PCR thermal cycler (Eppendorf, Hamburg, Germany) began with an initial denaturation step at 96°C for 4 minutes. Then, it was followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds according to specific primer requirements, and extension at 72°C for 60 seconds per kilobase of the target DNA sequence. After these

cycles, a final extension phase at 72°C for 10 minutes ensures the complete synthesis of all PCR products. The procedure concludes with a hold at 10°C to preserve the PCR products for further analysis with 10 µM 16s rRNA forward and reverse primers.

2.4 Analysis of PCR Products via Gel Electrophoresis

Following PCR amplification, the products were assessed using nucleic acid gel electrophoresis, a method facilitated by Electrophoresis Kits (BIO-RAD, CA, USA) according to the manufacturer's guidelines from Bio-Rad Inc. (2024), a technique that separates nucleic acid molecules like DNA or RNA by size, and distinguishing molecules up to approximately 20kb. For the agarose gel analysis, a 2% agarose gel was prepared using 2µL of nucleic acid stain and 1X TAE buffer in a gel tray 10 cm wide. Each analysis involved mixing 5µL of DNA sample with 1µL of loading dye, and a 1 KBP ladder was used for size determination. The gel was run at 100V for 40 minutes to ensure proper separation of the DNA fragments. The gel was analysed using Gel Doc™ EZ Imager (BIO-RAD, CA, USA) and Gel Doc™ EZ System (BIO-RAD, CA, USA).

2.5. PCR Product Purification using ExoSAP-IT™ Reagent

To purify the PCR products before sequencing, ExoSAP-IT™ (ThermoFisher, MA, USA) was utilized following the specific guidelines provided by the manufacturer (ThermoFisher, MA, USA). In the procedure, 5 µL of the post-PCR reaction product was mixed with 2 µL of ExoSAPIT™ reagent, resulting in a total reaction volume of 7 µL. The mixture was placed in the Mastercycler® nexus PCR thermal cycler (Eppendorf, Hamburg, Germany). The thermal cycling protocol involved an initial incubation at 37°C for 15 minutes to degrade residual primers and nucleotides, followed by a 15-minute incubation at 80°C to deactivate the ExoSAP-IT™ reagent. Subsequently, the purified PCR products were submitted to the Australian Genome Research Facility (AGRF) for Sanger sequencing analysis.

2.6. Conduct Antimicrobial resistance test (AMR) for isolated skin bacteria of samples

In the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol (Hudzicki, 2009), a modified approach was used to assess antimicrobial susceptibility in marine organisms. The testing medium consisted of a 3.33% Marine Broth 2216 with 2% agar, on which sterile inoculating loops were used to transfer 4 isolated colonies directly onto fresh marine agar plates, bypassing the typical saline suspension to enhance organism growth. Each plate was carefully arranged with 4 different antibiotic discs and a central negative control disc. In total, 8 antibiotics (Sulfafurazole 300ug, Chloramphenicol 30ug, Tetracycline 30ug, Erythromycin 15ug, Ampicillin 10ug, Streptomycin 10ug, Penicillin 1unit, and Penicillin 0.5unit) with negative control (Blank disc) were tested and distributed evenly across two plates. Each configuration was replicated three times. Following incubation at 18°C for 36 hours, the zones of inhibition around each disc were measured with callipers.

2.7. Identification and Phylogenetic Analysis of Microbial Species Using 16S rRNA Sequences

16S rRNA gene sequences obtained from the Australian Genome Research Facility (AGRF) were delivered in FASTA file format. These sequences were utilized to identify microbial species through the NCBI BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) online platform, which facilitated the matching of our sequences with those in the database to determine the closest known relatives. Following species identification, the phylogenetic relationships between the identified microbial species were explored using Geneious Prime (Geneious Biologics 2024/version 0.5. https://www.geneious.com).

2.8. Analysis and Selection for Full DNA Sequencing Post-AMR Testing

Following AMR testing and subsequent microbial identification, the raw data were analysed to identify unique patterns within species of the same genus. This analysis aimed to discover distinct genetic and/or resistance characteristics that could be insight for

understanding marine organism antibacterial resistance mechanisms or phylogenetic lineage. Based on the findings from this initial analysis, 8 samples exhibiting the most unique patterns were selected for full DNA sequencing.

2.9. Full DNA Sequencing and Resistance Gene Analysis Using MinION™ Mk1B and Bioinformatics Tools

Microbial cultures that showed antibiotic-resistant patterns were selected for whole genome sequencing. Libraries were prepared with the Nanopore Rapid Barcoding Kit (Rapid Barcoding Kit 24 V14 (SQK-RBK114.24)) and sequenced on the MinION (flow cell R10.4.1) (Oxford Nanopore, United Kingdom) (Jain et al., 2016). Guppy base calling converted raw signal to base pairs (Oxford Nanopore Technology). Data is available on the SRA under BioProject PRJNA1107358. Hybracter generated the contigs (Bouras et al., 2024), and the mean coverage of each contig was calculated for each genome. BLASTN was used for taxonomic classification, (Altschul et al., 1990) and Bakta for functional annotation of each genome (Schwengers et al., 2021). Full DNA sequencing of selected samples was conducted using the MinION™ Mk1B device from Oxford Nanopore Technologies (Jain et al., 2016), adhering to the manufacturer's protocol. Post-sequencing, the genomes were analysed to identify resistance genes using Patric, a tool within the BV-BRC (Bacterial and Viral Bioinformatics Resource Centre) (Olson et al., 2023). Additionally, a multiple assembly graph was generated to visualize the structure and connections within the genomic data using Bandage (Bioinformatics Application for Navigating De novo Assembly Graphs Easily) (Wick et al., 2015).

2.10. Raw AMR data statistical analysis

Additionally, the antimicrobial resistance (AMR) raw data associated with these identified microbes was subjected to statistical analysis using SPSS (IBM Corp., 2017), Multivariate test with Post-Hoc Tukey's test was conducted. In multivariate tests, 'Host vs Antibiotics' and 'Microbe vs Antibiotic' were compared and investigated significance. In the statistical analysis microbes were analysed in genera of the microbe and seven-gills shark was removed in host vs antibiotic analysis.

2.11. Bandage plot and BV-BRC for full DNA sequence genome annotation

For imaging full DNA sequence analysis, the Bioinformatics Application for Navigating De novo Assembly Graphs Easily (Bandage) program (Wick et al., 2015) was used for imaging full DNA sequence samples graph. In the BV-BRC (Olson et al., 2023), the full DNA sequenced samples target of gene was compared by Patric (Wattam et al., 2017) in BV-BRC antimicrobial gene target.

Chapter 3: Results

3.1. Microbial diversity across Elasmobranch hosts

In the experiment, 38 microbes from six hosts with 8 antibiotics were tested (Table 2). The 16S Sanger analysis of elasmobranchs skin microbes identified, 8 genera of bacteria are that included *Halomonas,* Marine bacterium*, Planococcus, Pseudoalteromonas, Psychrobacter, Shewanella, Stutzerimonas,* and *Vibrio* from 4 different hosts. Angel sharks (*Squatina australis*), based on 8 samples, predominantly hosted *Pseudoalteromonas* and *Psychrobacter*. Eagle rays (*Myliobatis tenuicaudatus*), with 10 samples, exhibited a mix of *Halomonas, Pseudoalteromonas, Stutzerimonas, Psychrobacter,* and Marine bacterium. Fiddler rays (*Trygonorrhina dumerilii*), represented by 9 samples, commonly hosted *Pseudoalteromonas* and Marine bacterium, with some presence of *Shewanella* and *Vibrio*. Melbourne skates (*Dentiraja cerva*), from 7 samples, primarily hosted *Pseudoalteromonas* and Marine bacterium. Port Jackson sharks (*Heterodontus portusjacksoni*), across 3 samples, exclusively hosted *Pseudoalteromonas*, while the Seven-gills shark (*Notorynchus cepedianus*), based on 1 sample, also hosted *Pseudoalteromonas*.

Table 2. Microbial Samples Collected from Elasmobranch Species in Gulf St Vincent, and Seacliff in Australia Provided by the Dinsdale Laboratory Team: Sample IDs, Host Types, Scientific Names, Microbe, Collection Locations, method, and Dates

Sample ID	Host	Scientific Name	Microbe	Collected Location	Collected Date	Collection Method
20	Angel shark	*Squatina australis*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
A01	Angel shark	*Squatina australis*	*Psychrobacter*	Gulf St Vincent, SA, Australia	24/05/2024	Skin Swab
A02	Angel shark	*Squatina australis*	*Psychrobacter*	Gulf St Vincent, SA, Australia	24/05/2024	Skin Swab
A03	Angel shark	*Squatina australis*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	24/05/2024	Skin Swab
A04	Angel shark	*Squatina australis*	Marine bacterium	Gulf St Vincent, SA, Australia	24/05/2024	Skin Swab
A10	Angel shark	*Squatina australis*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	24/05/2024	Skin Swab
1	Eagle Ray	*Myliobatis tenuicaudatus*	*Halomonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
3	Eagle Ray	*Myliobatis tenuicaudatus*	*Halomonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
4	Eagle Ray	*Myliobatis tenuicaudatus*	*Halomonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
5	Eagle Ray	*Myliobatis tenuicaudatus*	*Halomonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
6	Eagle Ray	*Myliobatis tenuicaudatus*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
14	Eagle Ray	*Myliobatis tenuicaudatus*	*Stutzerimonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
ER1	Eagle Ray	*Myliobatis tenuicaudatus*	*Planococcus*	Seacliff, SA, Australia	23/06/2022	Skin Swab
ER2	Eagle Ray	*Myliobatis tenuicaudatus*	Marine bacterium	Seacliff, SA, Australia	23/06/2022	Skin Swab
ER3	Eagle Ray	*Myliobatis tenuicaudatus*	Marine bacterium	Seacliff, SA, Australia	23/06/2022	Skin Swab
ER4	Eagle Ray	*Myliobatis tenuicaudatus*	*Pseudoalteromonas*	Seacliff, SA, Australia	23/06/2022	Skin Swab
ER5	Eagle Ray	*Myliobatis tenuicaudatus*	Marine bacterium	Seacliff, SA, Australia	23/06/2022	Skin Swab
ER6	Eagle Ray	*Myliobatis tenuicaudatus*	*Psychrobacter*	Seacliff, SA, Australia	23/06/2022	Skin Swab
16	Fiddler Ray	*Trygonorrhina dumerilii*	Marine bacterium	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
17	Fiddler Ray	*Trygonorrhina dumerilii*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
18	Fiddler Ray	*Trygonorrhina dumerilii*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
19	Fiddler Ray	*Trygonorrhina dumerilii*	*Shewanella*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker

24	Fiddler Ray	*Trygonorrhina dumerilii*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
A05	Fiddler Ray	*Trygonorrhina dumerilii*	Marine bacterium	Gulf St Vincent, SA, Australia	24/05/2024	Skin Swab
A06	Fiddler Ray	*Trygonorrhina dumerilii*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	24/05/2024	Skin Swab
A08	Fiddler Ray	*Trygonorrhina dumerilii*	*Vibrio*	Gulf St Vincent, SA, Australia	24/05/2024	Skin Swab
A12	Fiddler Ray	*Trygonorrhina dumerilii*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	24/05/2024	Skin Swab
7	Melbourne Skate	*Dentiraja cerva*	*Stutzerimonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
9	Melbourne Skate	*Dentiraja cerva*	Marine bacterium	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
10	Melbourne Skate	*Dentiraja cerva*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
11	Melbourne Skate	*Dentiraja cerva*	Marine bacterium	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
12	Melbourne Skate	*Dentiraja cerva*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
13	Melbourne Skate	*Dentiraja cerva*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
15	Melbourne Skate	*Dentiraja cerva*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
21	Port Jackson Shark	*Heterodontus portusjacksoni*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
22	Port Jackson Shark	*Heterodontus portusjacksoni*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
23	Port Jackson Shark	*Heterodontus portusjacksoni*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	15/02/2023	Skin Supersucker
25	Seven-gills shark	*Notorynchus cepedianus*	*Pseudoalteromonas*	Gulf St Vincent, SA, Australia	24/05/2024	Skin Swab

3.2. Statistical analysis of AMR test of isolated microbes to antibiotics

The statistical analysis of the antimicrobial sensitivity of isolated microbes to eight antibiotics showed significant variation in sensitivity across microbes (Figure 2). The multivariate test revealed a significant effect of antibiotics on microbes, as indicated by Pillai's Trace ($F(df = 56, 735) = 2.131$, $p < 0.001$) and Wilks' Lambda ($F(df=56, 538.442) = 2.287$, $p < 0.001$). Test of between-subject effects for specific antibiotics determined significant differences for Chloramphenicol ($F(df=7, 106) = 2.164$, $p = 0.043$), Tetracycline ($F(df=7, 106) = 2.940$, $p = 0.007$), Streptomycin ($F(df=7, 106) = 3.470$, $p =$

0.002), Penicillin (1 unit) ($F(df=7, 106) = 2.111$, $p = 0.049$), and Penicillin (0.5 unit) ($F(df=7, 106) = 2.529$, $p = 0.019$), meaning that sensitivity to these antibiotics varies significantly across the microbial genera. Post-hoc Tukey tests resulted in significant differences between specific genera for certain antibiotics. *Stutzerimonas* consistently showed higher sensitivity compared to other microbes for Tetracycline, Erythromycin, Streptomycin, Penicillin (1 unit), and Penicillin (0.5 unit). There were no statistically significant differences for Sulfafurazole, Ampicillin, and Chloramphenicol among the microbial groups.

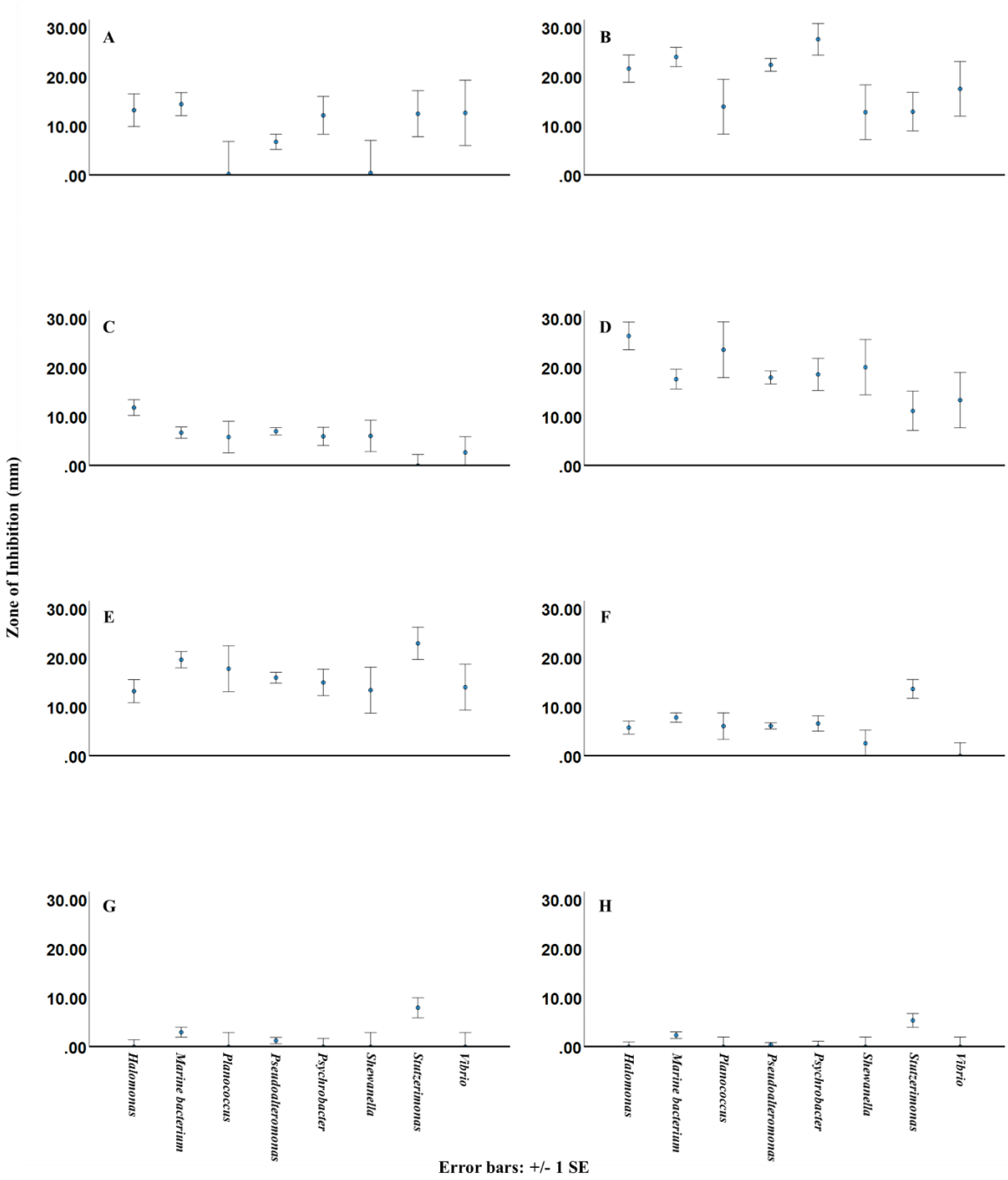


Figure 2. Mean Antimicrobial sensitivity (mm) across bacterial genus (Error bars +/- 1(SE)). Theses multi-plot graphs illustrate the mean inhibition zone diameters (mm) for eight different antibiotics (A: Sulfafurazole, B: Chloramphenicol, C: Tetracycline, D: Erythromycin, E: Ampicillin, F: Streptomycin, G: Penicillin1unit, H: Penicillin0.5unit)tested on various bacterial genus: Underline. Higher mean values indicate greater sensitivity to the antibiotics, while zero indicates resistance.

Psychrobacter exhibited the highest sensitivity (27.61mm) in Chloramphenicol. Tetracycline with *Halomonas* displayed the highest zone of inhibition (11.80mm) and *Stutzerimonas* showed resistance (0mm). Streptomycin with *Stutzerimonas* revealed the highest sensitivity (13.64mm). Erythromycin with *Halomonas* showed the highest sensitivity (26.41mm).

The highest resistance is observed for Penicillin 1 unit and Penicillin 0.5 unit, both with approx. 84% resistance in 38 microbes. However, Sulfafurazole, Chloramphenicol, Tetracycline, and Erythromycin show very low resistance (approx. 5% for Sulfafurazole and Tetracycline, and approx. 2% for Streptomycin). Chloramphenicol, Erythromycin, and Ampicillin are effective treatments for 38 microbe species (Figure 3).

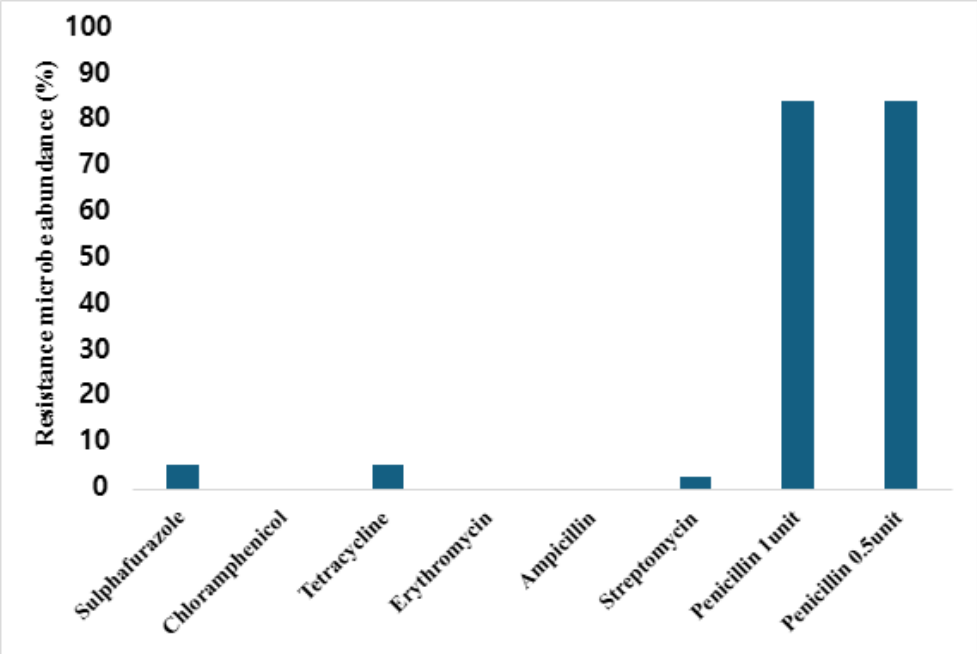


Figure 3. Summary of antibiotic resistance in 38 microbes across eight antibiotics

3.3. Statistical analysis of AMR test of elasmobranch hosts to antibiotics

The statistical analysis of antibiotic sensitivity across different elasmobranch hosts indicated significant differences in the effect of antibiotics (Figure 4). The multivariate test narrated a significant effect of host species on antibiotic sensitivity, as indicated by Pillai's Trace ($F(df=32, 408) = 3.853$, $p < 0.001$), suggesting that the host species significantly influenced microbial sensitivity to antibiotics. There was a significant effect of host type on resistance for Chloramphenicol ($F(df=4, 106) = 2.569$, p 0.042), Tetracycline ($F(df=4, 106) = 4.739$, $p = 0.001$), Erythromycin ($F(df=4, 106) = 16.799$, $p < 0.001$), Penicillin 1 unit ($F(df=4, 106) = 4.269$, $p = 0.003$), and Penicillin 0.5 unit ($F(df=4, 106) = 3.326$, $p = 0.013$). Post-hoc Tukey tests showed that Eagle Rays (*Myliobatis tenuicaudatus*) exhibited significantly higher sensitivity to Erythromycin compared to Angel Sharks (*Squatina australis*) ($p < 0.001$) and Melbourne skates (*Dentiraja cerva*) ($P < 0.001$). However, for antibiotics such as Sulfafurazole, the differences among hosts were not statistically significant ($p > 0.05$). The data showed that different host species exhibit varying degrees of sensitivity or resistance to the tested antibiotics, with significant differences highlighted in the Tukey test results.

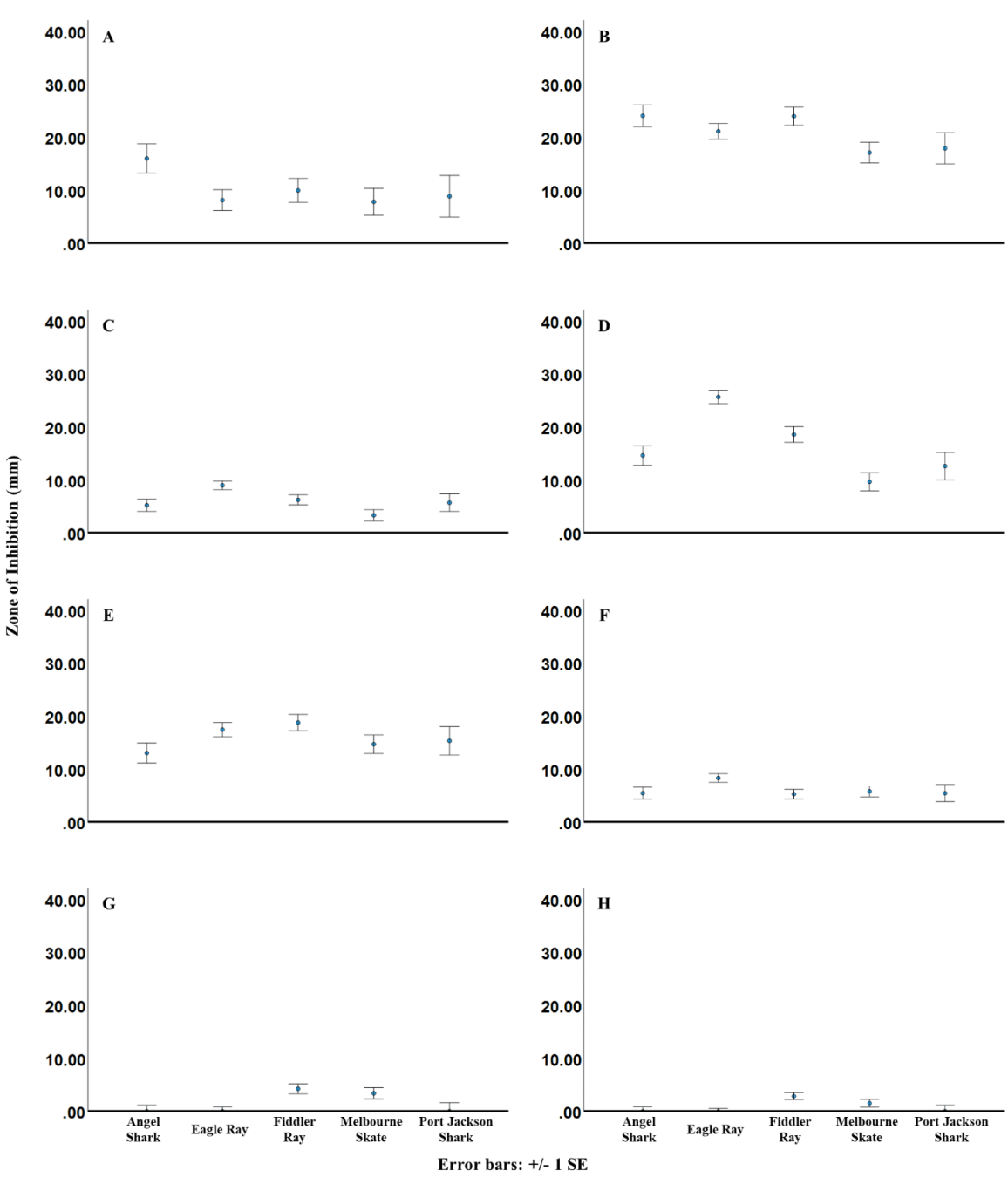


Figure 4. Comparison of mean antibiotic sensitivity (mm) across Elasmobranch species with Standard Error bars (SE). These multi-plot graphs show the mean inhibition zone diameters (mm) for eight different antibiotics (A: Sulfafurazole, B: Chloramphenicol, C: Tetracycline, D: Erythromycin, E: Ampicillin, F: Streptomycin, G: Penicillin1unit, H: Penicillin0.5unit) tested on microbial isolates from five elasmobranch species: Angel shark (Squatina australis), Eagle Ray

(Myliobatis tenuicaudatus), Fiddler Ray (Trygonorrhina dumerilii), Melbourne Skate (Dentiraja cerva), and Port Jackson Shark (Heterodontus portusjacksoni). Error bars represent the SE for each species, providing an estimate of variability around the mean. Higher mean values indicates greater sensitivity to antibiotics, while zero indicate resistance.

Erythromycin showed the highest mean sensitivity observed in Eagle Rays (*Myliobatis tenuicaudatus*) (25.66mm) and the lowest observed in Melbourne skates (*Dentiraja cerva*) (9.65mm). Chloramphenicol with Angel Sharks (*Squatina australis*) resulted in the highest sensitivity (24.07mm) and Melbourne skates (*Dentiraja cerva*) showed the lowest (17.10mm). In Tetracycline, Eagle Rays (*Myliobatis tenuicaudatus*) displayed the highest sensitivity (8.98mm). Ampicillin determined no significant differences among hosts, although Fiddler rays (*Trygonorrhina dumerilii*) showed the highest mean (18.78mm).

3.4. Phylogenetic relationship of isolated microbes

The close clustering of *Pseudomonas* and *Psychrobacter* species, particularly those isolated from Angel Sharks (*Squatina australis*) and Eagle Rays (*Myliobatis tenuicaudatus*), suggested these bacteria have similar traits, which may be adaption to their elasmobranch hosts. This may indicate a specialized shark-associated microbiome, where certain bacterial species may have co-evolved or become uniquely suited to thrive in the skin environment of these marine animals. For instance, *Psychrobacter* species isolated from Angel Sharks (*Squatina australis*) cluster closely together, suggesting a specialization to this host. Similarly, several *Pseudomonas* strains from Eagle Rays (*Myliobatis tenuicaudatus*) and Angel Sharks (*Squatina australis*) are also closely related,

reinforcing the idea of host-specific microbial adaptation within these elasmobranchs (Figure 5).

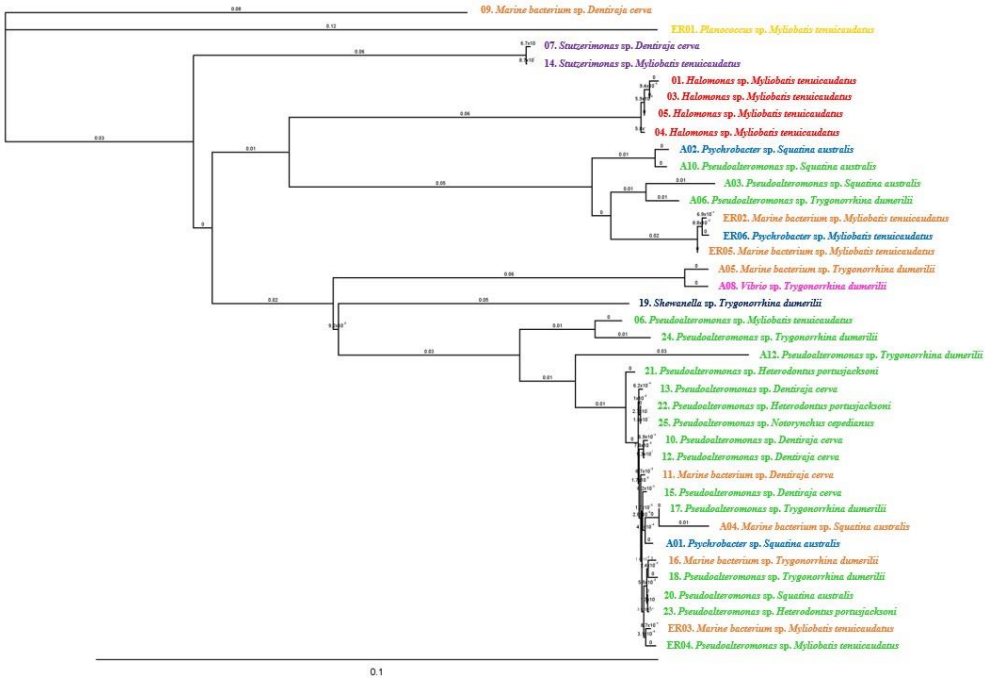


Figure 5. Phylogenetic relationship across 38 microbes from five host species. Microbe species are sorted by colour (Red: *Halomonas*, Orange: Marine bacterium, Yellow: *Planococcus*, Green: *Pseudoalteromonas*, Blue: *Psychrobacter*, Navy: *Shewanella*, Purple: *Stutzerimonas*, and Pink: *Vibrio*)

Stutzerimonas species from Melbourne skates (*Dentiraja cerva*) and Eagle Rays (*Myliobatis tenuicaudatus*) group with the reference strain *Stutzerimonas stutzeri* from NCBI database, indicating they were likely generalist environmental microbes rather than specifically adapted to elasmobranchs. On the other hand, bacteria such as *Planococcus* and Marine bacterium from Melbourne skates (*Dentiraja cerva*) form distinct branches, which, despite sharing similarities with reference strains, may represent unique

adaptations to the host environment or distinct evolutionary lineages, hinting at some degree of specialization (Figure 6).

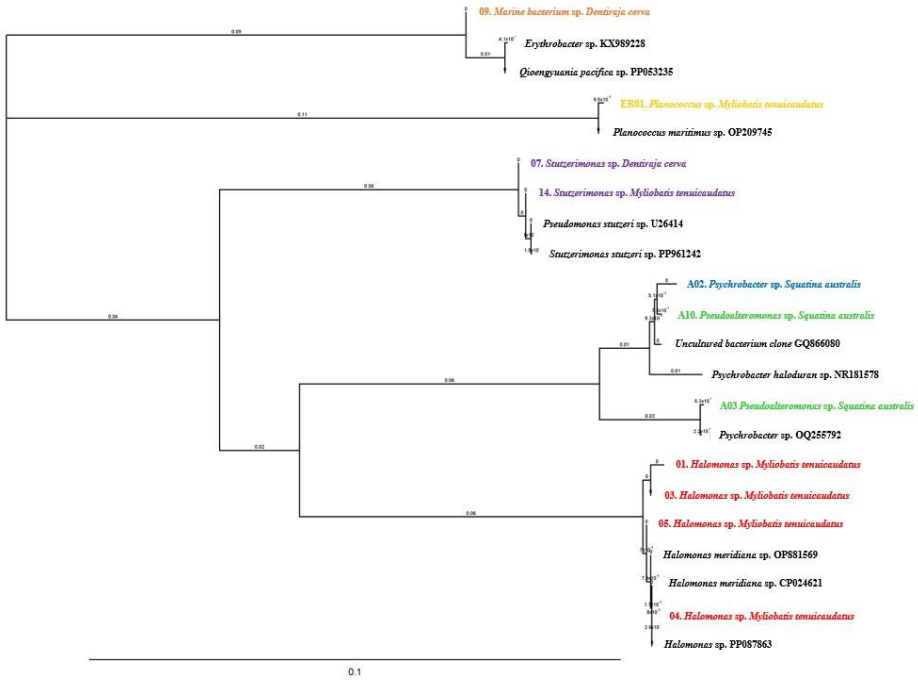


Figure 6. Phylogenetic Tree of Elasmobranch-Associated Bacteria with NCBI Reference Strains (Red: *Halomonas*, Orange: Marine bacterium, Yellow: *Planococcus*, Green: *Pseudoalteromonas*, Blue: *Psychrobacter*, and Black: NCBI Reference strain)

Shewanella sp. from Fiddler rays (*Trygonorrhina dumerilii*) closely groups with the reference strain *Shewanella sp. KT385867*, indicating it could be an environmental generalist adapted to the broader marine ecosystem. Conversely, *Vibrio sp.* isolated from Fiddler rays (*Trygonorrhina dumerilii*) forms a distinct clade, implying a unique adaptation or evolutionary lineage (Figure 7).

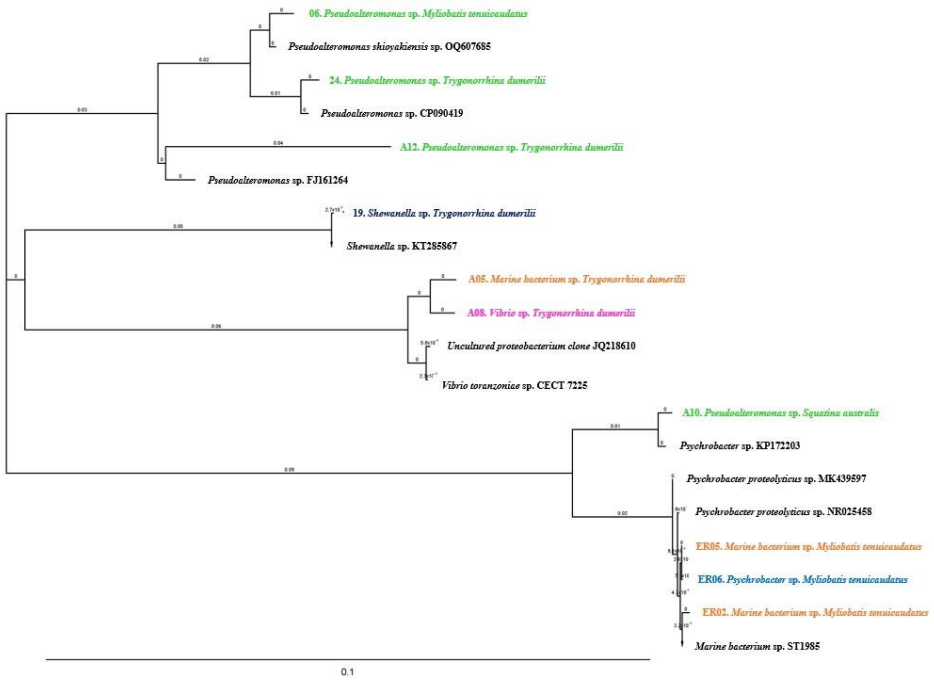


Figure 7. Phylogenetic Tree of Elasmobranch-Associated Bacteria with NCBI Reference Strains (Orange: Marine bacterium, Green: *Pseudoalteromonas*, Blue: *Psychrobacter*, Navy: *Shewanella*, Pink: *Vibrio* and Black: NCBI Reference strain)

Pseudoalteromonas and Marine bacterium isolates from elasmobranchs, such as Fiddler rays (*Trygonorrhina dumerilii*), Angel Sharks (*Squatina australis*), Port Jackson sharks (*Heterodontus portusjacksoni*), and Eagle Rays (*Myliobatis tenuicaudatus*), were similar to the reference strains from the NCBI database. Several *Pseudoalteromonas* species from elasmobranchs cluster closely with reference strains, such as *Pseudoalteromonas tetraodonis* and *Pseudoalteromonas agarivorans*, suggesting that these bacteria are generalist marine microbes, widely distributed in the ocean environment (Figure 8).

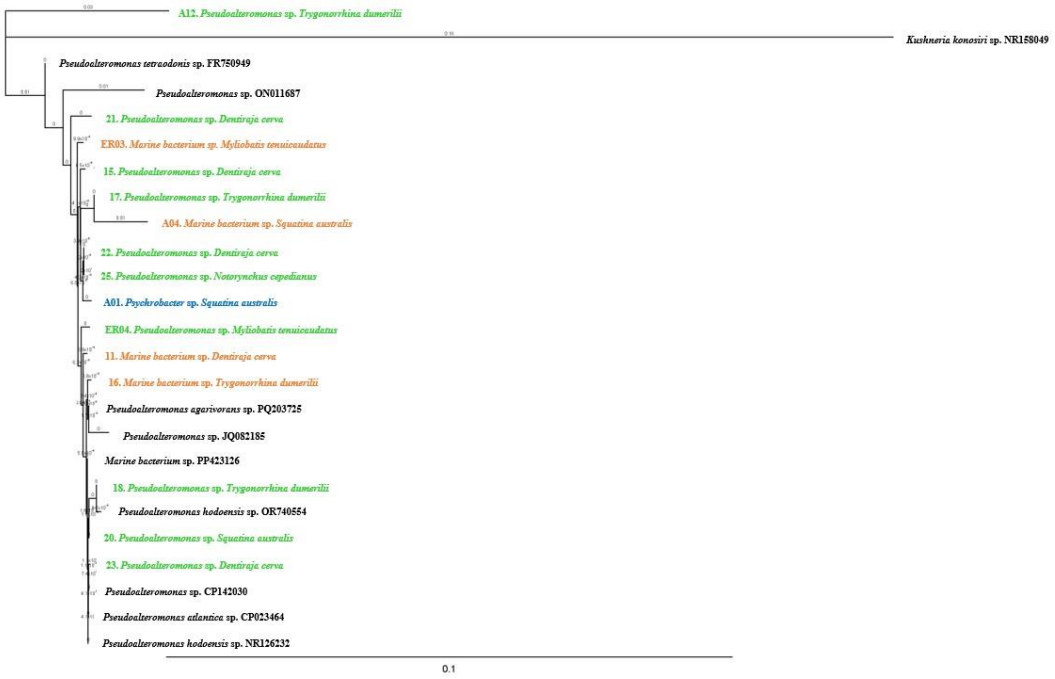


Figure 8. Phylogenetic Tree of Elasmobranch-Associated Bacteria with NCBI Reference Strains (Orange: Marine bacterium, Green: *Pseudoalteromonas*, Blue: *Psychrobacter*, and Black: NCBI Reference strain)

3.5. Analysis of full sequence genomes via BV-BRC

The data analysis of full sequence DNA via BA-BRC genomic annotation, showed variability in bacterial isolates from elasmobranch hosts, with *Pseudoalteromonas sp.* from Fiddler rays (*Trygonorrhina dumerilii*) having both incomplete genomes (242 contigs, 4.18 Mb) and complete genomes (5 contigs, 4.38 Mb). In contrast, Marine bacterium sp. from Angel Sharks (*Squatina australis*) has a complete genome (2 contigs, 4.76 Mb), while isolates from Eagle Rays (*Myliobatis tenuicaudatus*), including Marine bacterium sp., have incomplete genomes with 2 contigs and genome lengths ranging from 4.29 Mb to 4.46 Mb. *Psychrobacter sp.* from an Eagle Rays (*Myliobatis tenuicaudatus*) has a complete genome with 1 contig and a genome length of 3.29 Mb Samples from Eagle Ray (Bacteria ER02, Bacteria ER03, and Bacteria ER04) showed identical microbes (*Pseudoalteromonas espejiana*) (Table 3).

Table 3. Genome Annotation of nanopore samples by BV-BRC. The table outlines the genomic characteristics of bacterial species isolated from different hosts, sample identifiers, host species, associated microbes, the number of contigs assembled, genome length, genome quality classification, and the closest matching genomes from the BV-BRC database with quality scores

Sample ID	Host	Contigs	Genome Length	Genome Quality	Similar Genome from BV-BRC	Genome Quality Coarse
17	Fiddler Ray	242	4183895	Incomplete	*Pseudoalteromonas agarivoran*s strain Hao 2018	92.53
A12	Fiddler Ray	5	4382437	Complete	*Pseudoalteromonas tetraodonis* strain GFC	99.2
A05	Fiddler Ray	17	2441997	Incomplete	*Psychrobacter celer* strain DSM 23510	94.27
A04	Angel shark	2	4767950	Complete	*Vibrio toranzoniae* strain CECT 7225	98.7
ER06	Eagle Ray	1	3290539	Complete	*Psychrobacter nivimaris* strain Psychrobacter nivimaris 88-2-7	94.39
ER05	Eagle Ray	2	4462122	Incomplete	*Pseudoalteromonas espejiana* strain ATCC 29659	98.2
ER03	Eagle Ray	2	4293194	Incomplete	*Pseudoalteromonas espejiana* strain ATCC 29659	98.2
ER04	Eagle Ray	78	4411157	Incomplete	*Pseudoalteromonas espejiana* strain ATCC 29659	98.2

The Phylogenetic tree generated from BV-BRC presented the genetic identity distance among fully sequenced genome samples, as indicated by bootstrap values of 100 at all nodes, supporting high confidence in tree topology. Bacteria ER02 *Pseudoalteromonas* sp. from Fiddler ray (*Trygonorrhina dumerilii*), Bacteria ER02, Bacteria ER03, and Bacteria ER04 were closely related and Bacteria ER01 and Barcode06 were distinct separate lineages (Figure 9). The alignment of 12,400 amino acids and 37,200 nucleotides provides a robust dataset. Despite 16S rRNA similarities, the full genome data reveal distinct phylogenetic groupings.

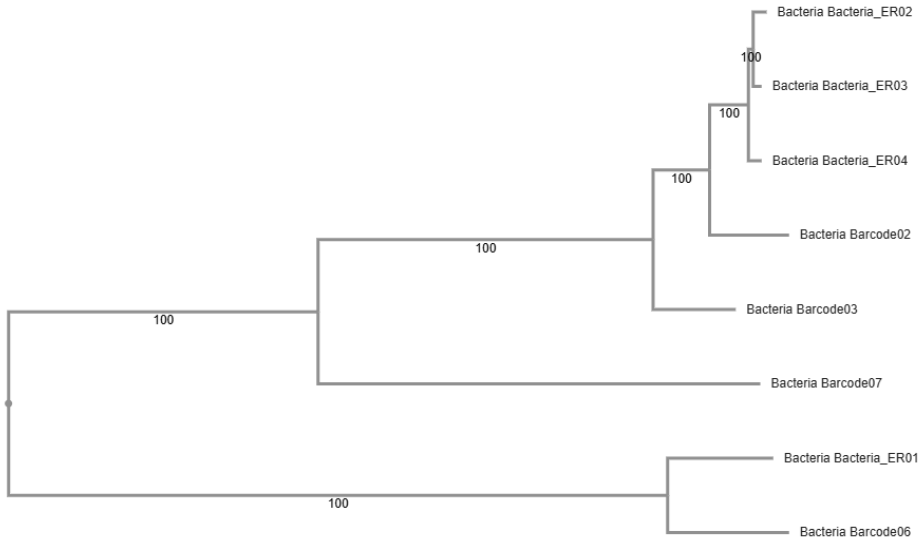


Figure 9. Phylogenetic analysis of bacterial samples using whole-genome sequencing from BV-BRC Bacteria02 *Pseudoalteromonas* sp., Bacteria03 *Pseudoalteromonas* sp., Bacteria 06 Marine bacterium sp., Bacteria 07 Marine bacterium sp., Bacteria ER01 *Psychrobacter* sp., Bacteria ER02 Marine bacterium sp., Bacteria ER03 *Pseudoalteromonas* sp., Bacteria ER04 *Pseudoalteromonas* sp. Bootstrap values (100) indicate strong support for the branch topology, suggesting other genomic regions are driving the observed phylogenetic structure

Bacteria ER02 and Bacteria ER03 form a clade with a bootstrap value of 92, showing closely related to *Pseudoalteromonas espejiana* strain ATCC 29659. Bacteria ER04 showed a separated branch. Barcode02 and *Pseudoalteromonas agarivorans* strain Hao 2018 from BV-BRC reference database were clustered together, supported by a bootstrap value of 100 (Figure 10). The large number of aligned amino acids (21,703) and nucleotides (65,109) supported the reliability of phylogenetic relationship.

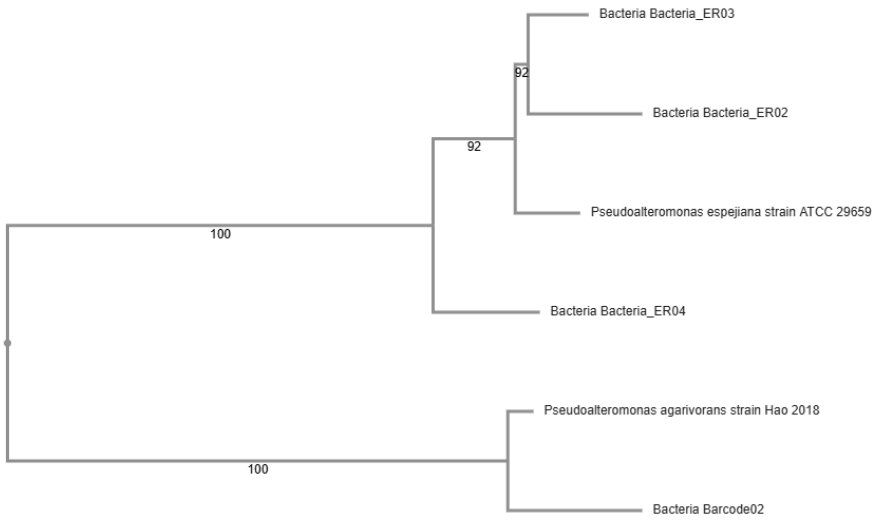


Figure 10. Phylogenetic tree generated from full genome sequencing, showing the relationships between environmental isolates (Bacteria_ER02, Bacteria_ER03, Bacteria_ER04, and Bacteria Barcode02) and known *Pseudoalteromonas* species. Bootstrap values (100, 92) indicate strong to moderate support for the groupings.

Full sequence samples successfully matched to known species from BV-BRC reference databases. Bacteria ER01 clusters with *Psychrobacter nivimaris* sp. and *Psychrobacter celer* sp., indicating a strong phylogenetic link to the *Psychrobacter* genus. Barcode 06 is closely related to *Psychrobacter* species, reinforcing its identification. Similarly, Barcode 03 clusters with *Pseudoalteromonas tetraodonis* strain GFC, while Barcode 07 matches with *Vibrio toranzoniae* strain CECT7225, confirming the correct identification of these barcodes with known strains (Figure 11).

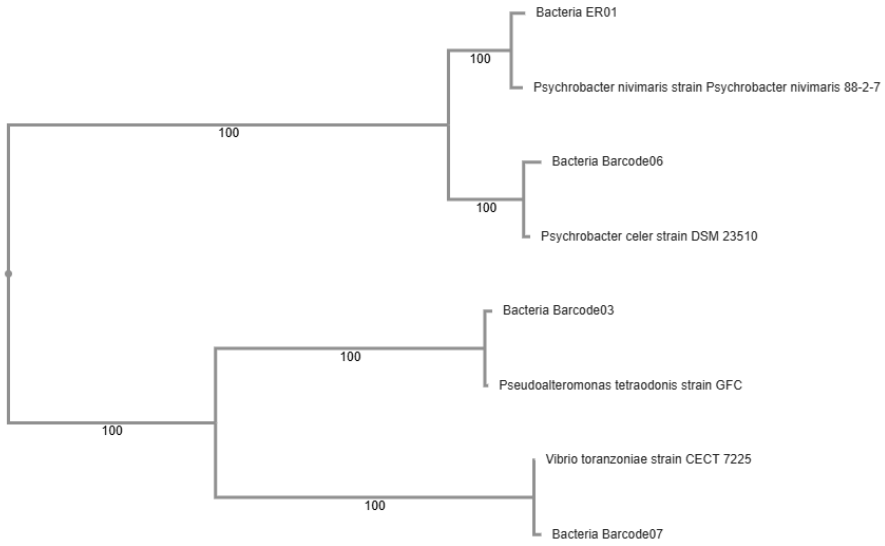


Figure 11. Phylogenetic tree generated using BV-BRC, illustrating the evolutionary relationships between Bacteria_ER01, *Psychrobacter* species, *Pseudoalteromonas* species, *Vibrio toranzoniae*, and other related strains. All nodes are supported by bootstrap values of 100, indicating high confidence in the tree's structure. The tree is based on an alignment of 27,110 amino acids and 81,330 nucleotides. All nodes have bootstrap values of 100, demonstrating strong support for the relationships shown.

3.6. Analysis of Antibiotic Resistance Genes

In the analysis of ARGs identified via BV-BRC 'Specialty Genes' and experiment antibiotic susceptibility testing, several matches and inconsistencies were observed. Barcode 06 *Pseudoalteromonas* sp. reveals folP and S10p genes in BV-BRC, yet only Tetracycline shows 0mm inhibition in experimental data, with no Sulfafurazole test result, indicating incomplete validation. Barcode 02 *Pseudoalteromonas* sp. shows MexEF-OprN, TolC/OpmH, and folP genes, but experimentally Penicillin exhibited 0mm inhibition, which does not consistency directly with predicted resistances. In Barcode 03 *Pseudoalteromonas* sp., resistance genes expected for Chloramphenicol, Tetracycline,

and Streptomycin (MexEF-OprN, TolC/OpmH, S10p, S12p) show no experimental inhibition to Penicillin, Sulfafurazole, or Tetracycline, supporting some BV-BRC database. Barcode 07 *Marine bacterium* sp. lists resistance to Tetracycline (TolC/OpmH, Tet(35)) and beta-lactamase for Penicillin and Ampicillin, which is consistent with the experimental result of 0mm inhibition for these antibiotics (Table 4). Commonly across samples, tetracycline resistance (via S10p or TolC/OpmH) is frequently found in PATRIC database, with consistent 0mm inhibition in experimental data, however, mismatches in other antibiotics (e.g., Sulfafurazole, beta-lactams) suggest variations in gene expression or experimental limitation in detecting all predicted resistance.

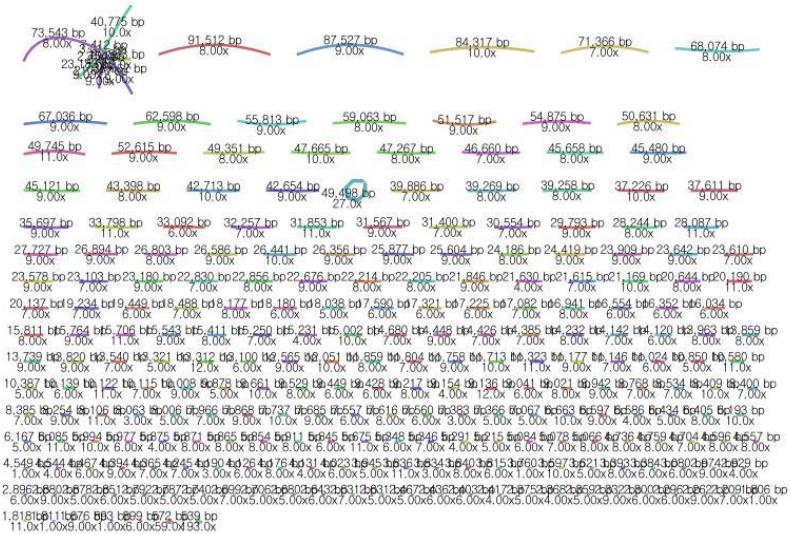
Table 4. Comparison of potential ARGs from BV-BRC genome annotation with AMR experiment data

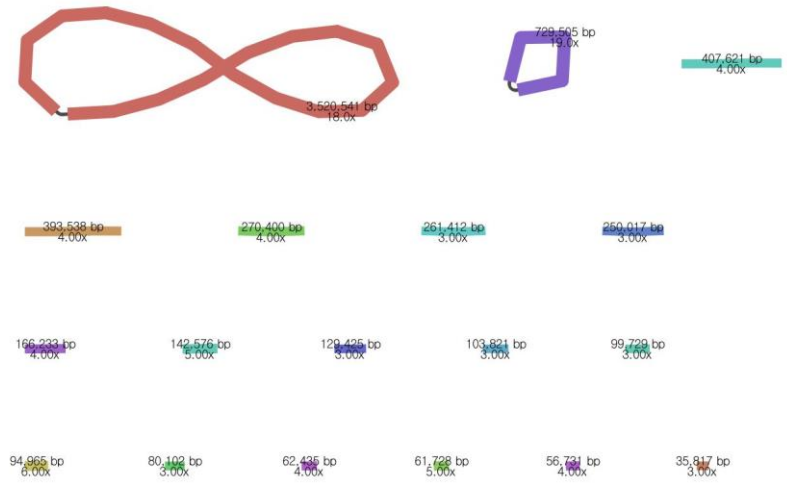
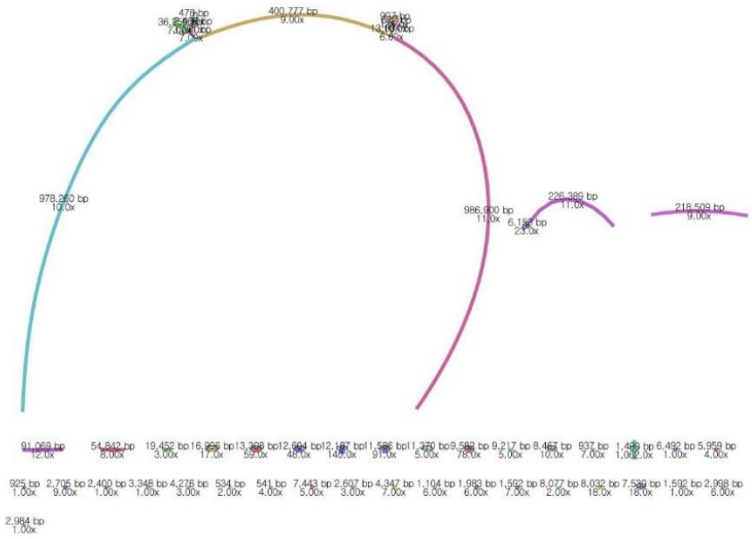
Host	Microbe	Patric ARG no.	Actual AMR no.	Type of Antibiotic and ARGs from PATRIC Specialty Genes (Potential gene)	Type of Resistance Antibiotic from Experiment and Zone of Inhibition (mm)
Barcode 02) Fiddler Ray	***Pseudoalteromonas* sp.**	59	2	Efflux MexEF-OprN (**Chloramphenicol**) TolC/OpmH (**Tetracycline**) S10p (**Tetracycline**) S12p (**Streptomycin**) folP (**Sulfafurazole**)	**Penicillin 1unit: 0mm** **Penicillin 0.5unit: 0mm**
Barcode 03) Fiddler Ray	***Pseudoalteromonas* sp.**	53	4	Efflux MexEF-OprN (**Chloramphenicol**) TolC/OpmH (**Tetracycline**) S10p (**Tetracycline**) S12p (**Streptomycin**)	**Sulfafurazole: 0mm** **Tetracycline: 0mm** **Penicillin 1unit: 0mm** **Penicillin 0.5unit: 0mm**
Barcode 06) Fiddler Ray	**Marine bacterium sp.**	51	1	folP (**Sulfafurazole**) S10p (**Tetracycline**)	**Tetracycline: 0mm**
Barcode 07) Angel shark	**Marine bacterium sp.**	51	4	TolC/OpmH (**Tetracycline**) Tet(35) (**Tetracycline**) Class A beta-lactamase and Class C beta-lactamase (**Penicillin and Ampicillin**) folP (**Sulfafurazole**)	**Sulfafurazole: 0mm** **Ampicillin: 0mm** **Penicillin 1unit: 0mm** **Penicillin 0.5unit: 0mm**
BacteriaER01) Eagle Ray	***Psychrobacter* sp.**	41	3	Efflux MacA and MacB (**Erythromycin**) S10p(**Tetracycline**) S12p (**Streptomycin**) gidB (**Streptomycin**) Class C beta-lactamase (**Penicillin and Ampicillin**) folP (**Sulfafurazole**)	**Tetracycline: 0mm**
BacteriaER02) Eagle Ray	**Marine bacterium sp.**	61	2	Efflux MexEF-OprN (**Chloramphenicol**) TolC/OpmH (**Tetracyclines**) S10p (**Tetracycline**) folP (**Sulfafurazole**)	**Penicillin 1unit: 0mm** **Penicillin 0.5unit: 0mm**
BacteriaER03) Eagle Ray	**Marine bacterium sp.**	48	2	Efflux MexEF-OprN (**Chloramphenicol**) TolC/OpmH (**Tetracyclines**) S10p (**Tetracycline**) folP (**Sulfafurazole**)	**Penicillin 1unit: 0mm** **Penicillin 0.5unit: 0mm**
BacteriaER04) Eagle Ray	***Pseudoalteromonas* sp.**	56	3	Efflux MexEF-OprN (**Chloramphenicol)** TolC/OpmH (**Tetracyclines**) S10p (**Tetracycline**) Subclass B3 beta-lactamase (**Penicillin and Ampicillin**) folP (**Sulfafurazole**)	**Streptomycin: 0mm** **Penicillin 1unit: 0mm** **Penicillin 0.5unit: 0mm**

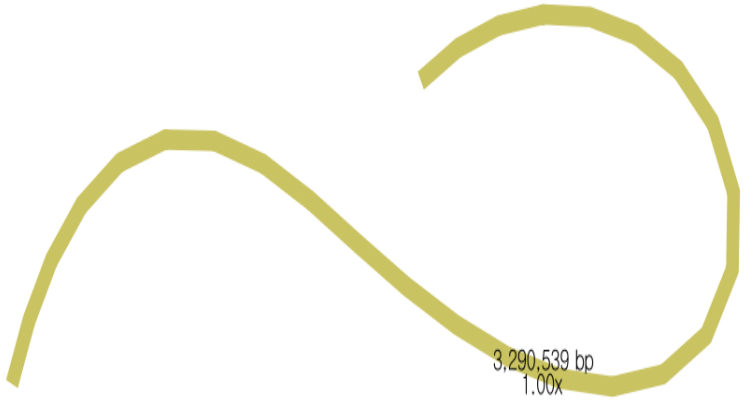
3.7. Analysis of DNA full sequenced sample graph via De novo Assembly Graphs Easily (Bandage) program

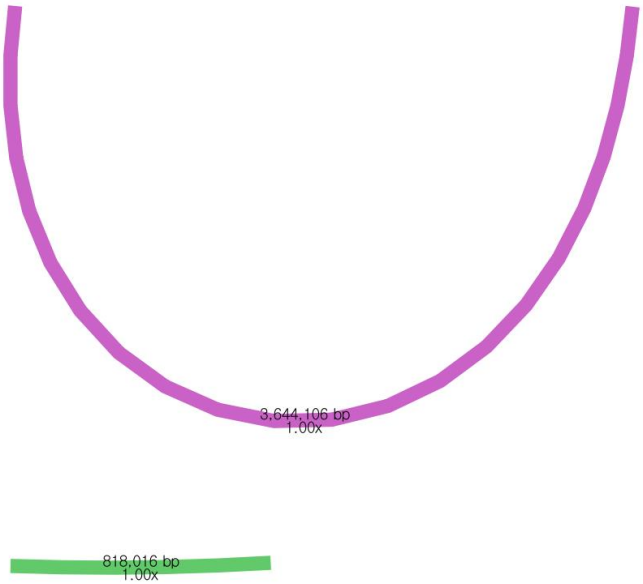
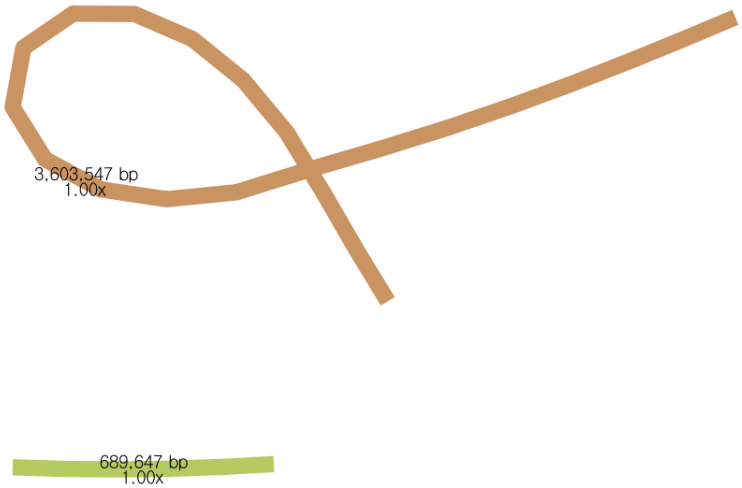
When comparing the complete genome to the incomplete ones, Overall structure and graph quality for complete assemblies are much simpler and more resolved. Complete genomes, such as Barcode 03, Barcode 07, and Bacteria ER01 have fewer nodes and edges, indicating fewer breaks or gaps in the assembly. These graphs have clearer, more contiguous structures, suggesting that the genome is almost fully assembled with very few unresolved regions. Circular or near-complete linear sequences, indicate a high level of confidence in the assembly process. However, incomplete genome, Barcode 02, Barcode 06, and Bacteria ER02 exhibit more fragmented structures, with the higher number of nodes and edges. These graphs show disjointed segments or contigs that have not been linked together due to low sequencing coverage, repetitive sequences, or complex genome structures (Table 5).

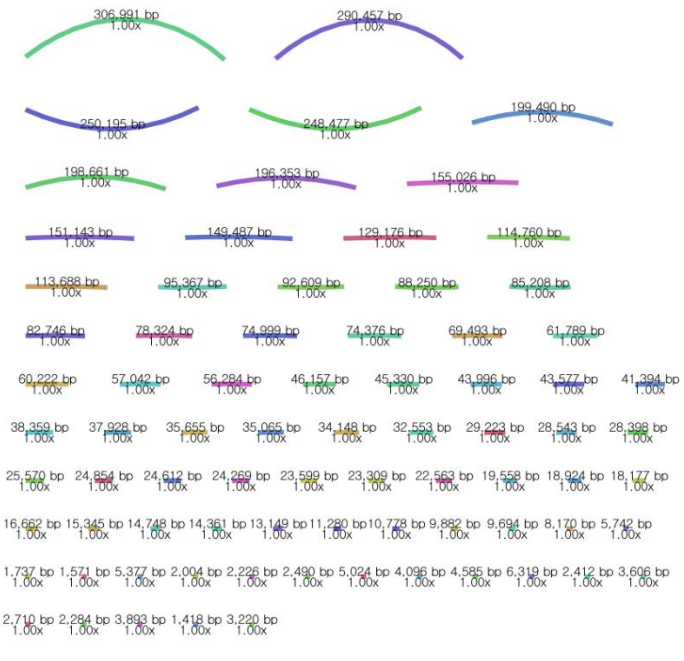
Table 5. Comparison of genome assembly graph from De novo Assembly Graphs Easily (Bandage) program for complete and incomplete genomes.

Sample ID	Genome quality and Graph size	Bandage graph
Barcode 02	Quality: Incomplete Node count: 248 Edge count: 12 Total length (no overlaps): 4,196,260bp	

Barcode 03	Quality: Complete Node count: 18 Edge count: 2 Total length (no overlaps): 6,866,596bp	
Barcode 06	Quality: Incomplete Node count: 48 Edge count: 24 Total length (no overlaps): 3,224,640bp	

Barcode 07	Quality: Complete Node count: 3 Edge count: 4 Total length (no overlaps): 4,760,171bp	
BacteriaER01	Quality: Complete Node count: 1 Edge count: 0 Total length (no overlaps): 3,290,539bp	

BacteriaER02	Quality: Incomplete Node count: 2 Edge count: 0 Total length (no overlaps): 4,462,122bp	
BacteriaER03	Quality: Incomplete Node count: 3 Edge count: 1 Total length (no overlaps): 4,424,926bp	

BacteriaER04	Quality: Incomplete Node count: 78 Edge count: 0 Total length (no overlaps): 4,411,157bp	

Chapter Four: Discussion

4.1. Microbes cultured from 6 elasmobranchs

Bacteria from eight microbial genera were isolated from the skin microbiome of six elasmobranch species, including *Halomonas,* Marine bacterium, *Planococcus, Pseudoalteromonas, Psychrobacter, Shewanella, Stutzerimonas,* and *Vibrio*. *Pseudoalteromonas* and *Psychrobacter* were found to be predominant across most hosts, especially in Angel Sharks (*Squatina australis*) and Eagle Rays (*Myliobatis tenuicaudatus*), suggesting these bacteria are well-adapted to the skin environment of these marine organisms. *Stutzerimonas* in Melbourne skates (*Dentiraja cerva*) and Fiddler rays (*Trygonorrhina dumerilii*), showed close distance in the phylogenetic tree, suggesting that some microbes might be generalists, capable of adapting in both host-associated and environmental contexts. However, other bacteria, like *Pseudoalteromonas* and *Psychrobacter*, formed tight clusters in phylogenetic trees, indicating they may have co-evolved with their elasmobranch hosts, potentially reflecting a specialized adaptation to the host environment.

4.2. Host-microbe interaction

The relationship between elasmobranch hosts and their microbial communities appears to be highly host-specific, with certain bacteria being more prevalent in particular species. The presence of *Pseudoalteromonas* in all host species studied suggests a common adaptation mechanism to the host skin, potentially providing benefits such as defence against pathogens (Rathinam et al., 2024). In contrast, *Psychrobacter*, observed primarily in Angel Sharks (*Squatina australis*), may have adapted to specific host conditions such as pH, salinity, and immune factors (Doane et al., 2020; Goodman et al., 2022). The significant impact of host species on antimicrobial resistance also supports the idea that the host environment plays a crucial role in determining the resistance patterns of its associated microbiota (Ritchie et al., 2017). This finding emphasizes the complexity of host-microbe interactions in the marine ecosystem and raises the question of whether host

immune responses and environmental factors contribute to the selective pressures that drive microbial resistance (Diwan et al., 2023).

4.3. Significance of Host vs. Antibiotics and Microbes vs. Antibiotics

Bacteria cultured from Eagle rays (*Myliobatis tenuicaudatus*) exhibited higher sensitivity to Erythromycin compared to other hosts, such as Angel sharks (*Squatina australis*) and Melbourne skates (*Dentiraja cerva*). Significant differences were observed in microbial sensitivity to various antibiotics based on the host species, suggesting that the host environment plays a crucial role in shaping microbial resistance. While host species showed sensitivity in most antibiotics during AMR testing, specific resistance to Penicillin was observed in Angel sharks (*Squatina australis*), Eagle ray (*Myliobatis tenuicaudatus*), and Port Jackson sharks (*Heterodontus portusjacksoni*). A recent study found that 62.5% of microbes in Gulf seawater are resistant to Penicillin due to pollution from industrial runoff, agricultural waste, and untreated sewage that introduce antibiotics and resistant bacteria (Krupesha & Sumithra, 2023). The antimicrobial-resistant bacteria (ARB) can be spread easily in aquatic environment (Okoye et al., 2022), and the Penicillin-resistant in elasmobranch host species may affected by environmental contamination. However, elasmobranch skin mucus can produce antibiotic-producing capabilities (Ritchie et al., 2017), the clear factor of Penicillin resistance hasn't been discovered yet and requires further study.

The host-associated microbiome of elasmobranchs skin microbiome displayed variation in antibiotic sensitivity across bacterial genera. *Stutzerimonas* which was only found in Melbourne skates and Eagle rays, showed consistently higher sensitivity to several antibiotics, including Ampicillin, Streptomycin, and Penicillin indicating its broad susceptibility to multiple antimicrobial agents with resistance only to Tetracycline. The rest of the seven microbes *Halomonas, Planococcus, Pseudoalteromonas, Psychrobacter, Shewanella, Vibrio,* and the most dominant microbe *Pseudoalteromonas* were resistant to Penicillin. Despite these patterns of resistance, statistical analysis of antibiotic resistance patterns revealed antibiotic resistance was not highly abundant across eight microbes tested. This suggests that the skin microbiomes of elasmobranchs in Australia

are generally sensitive to common antibiotics including Sulfafurazole, Chloramphenicol, Tetracycline, Erythromycin, Ampicillin, Streptomycin, indicating a relatively low abundance of resistance in these microbial communities.

4.4. Antibiotic Resistance Genes (ARGs) and inconsistencies with actual data

While the genomic analysis via BV-BRC identified multiple antibiotic resistance genes (ARGs) in the microbial isolates, there was a visible inconsistency between the predicted resistance based on BV-BRC ARGs and the actual experimental data from antimicrobial susceptibility tests. Such as *Pseudoalteromonas* sp. isolates from Fiddler rays (*Trygonorrhina dumerilii*) were predicted to have resistance genes for Efflux MexEF-OprN (Chloramphenicol), TolC/OpmH (Tetracycline), S10p (Tetracycline), S12p (Streptomycin), folP (Sulfafurazole), yet the experimental results showed resistance for Beta-lactamase (Penicillin 1unit and Penicillin 0.5unit (zone of inhibition (0mm)). This inconsistency between BV-BRC ARGs and the experimental database can be explained by considering multiple resistance mechanisms.

MexEF-OprN, are often expressed under specific conditions, such as the presence of antibiotics or other stressors. If the experimental conditions did not induce these stress factors, the genes may remain inactive if these triggers are absent (Lamarche & Deziel, 2011). Efflux pumps involving TolC may not always be the dominant mechanism of resistance, and TolC mutations in efflux pump components can lead to altered drug specificity (Kantarcioglu et al., 2024) and may be involved in removing other compounds from the bacterial cell. Gene expression can vary depending on environmental factors such as antibiotic exposure, nutrient availability, or stress conditions, which may not have been in my experimental setup (Ghosh et al., 2020).

This suggests that while ARGs are present, they may not always be expressed or functionally active, potentially due to regulatory mechanisms or gene mutations that were not detected in the cultured genomes (Nielsen et al., 2022). The inconsistency of gene content and lab experiments highlights the complexity of predicting antimicrobial resistance based only on genomic data and underscores the need for integrating both genomic and phenotypic analyses to fully understand microbial resistance (Hu et al.,

2024). Another possible explanation for the inconsistencies between the BV-BRC ARGs and my experimental data could be incomplete or lower coverage of sequencing data in samples (Lionel et al., 2018).

4.5. Key Findings

- **Penicillin Resistance**: Penicillin was largely ineffective against elasmobranch skin microbiomes, with resistance observed in 84% of tested microbes.
- **Low Resistance for Other Antibiotics**: Sulfafurazole, Chloramphenicol, Tetracycline, and Erythromycin showed low resistance rates (<4%), indicating that these antibiotics remain effective in these microbial communities.
- **Environmental Impact**: The low abundance of resistant bacteria suggests limited environmental contamination in the sampled areas, despite the presence of resistant bacteria in surrounding seawater.

These findings suggest that elasmobranch skin microbiomes in Australian waters have not yet developed widespread antibiotic resistance, although further studies are needed to confirm this in other regions and under varying environmental conditions.

4.6. Future direction

The use of functional genomics and metagenomics can provide deeper insights into microbial resistance mechanisms within the broader microbiome context. The Dinsdale lab is currently sequencing metagenomes from these host organisms, with which the cultured genomes can be directly compared. This comparison would allow for a more comprehensive understanding of how microbial communities respond to antibiotics and adapt to the host environment, revealing potential new targets for combating antimicrobial resistance.

4.7. Conclusion

My study highlights the diversity of microbes and antibiotic resistance patterns of six elasmobranch skin microbiomes, with *Pseudoalteromonas* and *Psychrobacter* being predominant. While the microbial communities generally showed high resistance to penicillin (~84%), other antibiotics such as Sulfafurazole, Chloramphenicol, Tetracycline, and Erythromycin remained effective. Only eight cultured microbes were fully sequenced, while the rest relied on 16S rRNA sequencing, which limits a complete understanding of the microbial genome. Inconsistencies between potential antibiotic resistance genes from BV-BRC analysis and antimicrobial susceptibility test results showed the complexity of resistance mechanisms. Further research involving complete genome sequencing across more microbial samples is needed to fully understand the genetic basis of antibiotic resistance and microbial adaptation in these marine organisms.

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Appendices

Table 6. Supplementary Table: Antibiotic Susceptibility (AMR) Test raw data

ID	Host	Microbe	Rep.	Sulfafurazole	Chloramphenicol	Tetracycline	Erythromycin	Ampicillin	Streptomycin	Penicillin1 unit	Penicillin 0.5unit	Negative Control
20	Angel Shark	*Pseudoalteromonas*	1	0	15.31	0	3.39	15.51	2.31	0	0	0
20	Angel Shark	*Pseudoalteromonas*	2	0	2.94	0	7.94	14.32	5.94	0	0	0
20	Angel Shark	*Pseudoalteromonas*	3	0	3.43	0	8.37	13.37	7.38	0	0	0
A01	Angel Shark	*Psychrobacter*	1	2.4	34.49	2.7	10.05	23.08	1.8	0	0	0
A01	Angel Shark	*Psychrobacter*	2	2.1	34.37	1.5	15.27	20.21	4.78	0	0	0
A01	Angel Shark	*Psychrobacter*	3	1.04	32.32	1.55	20.36	6.92	12.1	0	0	0
A02	Angel Shark	*Psychrobacter*	1	31.97	34.74	2.4	16.63	15.62	11.6	0	0	0
A02	Angel Shark	*Psychrobacter*	2	32.68	32.28	8.32	15.03	5.04	4.86	0	0	0
A02	Angel Shark	*Psychrobacter*	3	31.85	31.48	17.15	8.36	14.04	4.18	0	0	0
A03	Angel Shark	*Pseudoalteromonas*	1	32.76	36.51	4.32	4.74	21.04	4.07	0	0	0
A03	Angel Shark	*Pseudoalteromonas*	2	27.99	31.11	5.89	19.6	3.17	5.47	0	0	0
A03	Angel Shark	*Pseudoalteromonas*	3	35.84	29.57	1.85	7.93	9.87	3.55	0	0	0
A04	Angel Shark	*Marine bacterium*	1	33.24	18.99	8.22	8.11	14.33	8.87	0	0	0
A04	Angel Shark	*Marine bacterium*	2	17.08	12.68	8.11	3.75	8.34	7.72	0	0	0
A04	Angel Shark	*Marine bacterium*	3	36.63	23.06	10.11	10.67	11.66	9.84	0	0	0
A10	Angel Shark	*Pseudoalteromonas*	1	0.91	21.02	14.69	33.58	10.72	0	0	0	0
A10	Angel Shark	*Pseudoalteromonas*	2	0	21.05	4.31	32.07	12.15	0	0	0	0
A10	Angel Shark	*Pseudoalteromonas*	3	1.58	17.92	2.86	37.21	15.41	3.83	0	0	0
1	Eagle Ray	*Halomonas*	2	37.99	37.02	16.86	21.8	18.4	0	0	0	0
1	Eagle Ray	*Halomonas*	3	25.61	32.06	26.3	28.48	21.93	0	0	0	0
1	Eagle Ray	*Halomonas*	1	37.25	33.69	18.94	28.39	19.44	0	0	0	0
3	Eagle Ray	*Halomonas*	1	11.53	15.92	13.14	20.19	11.59	0	0	0	0
3	Eagle Ray	*Halomonas*	2	11.03	20.85	5.76	24.55	12.5	0	0	0	0
3	Eagle Ray	*Halomonas*	3	3.77	18.08	5.81	14.97	13.14	0	0	0	0
4	Eagle Ray	*Halomonas*	1	0	22.14	8.6	30.03	14.41	14.73	0	0	0
4	Eagle Ray	*Halomonas*	2	0	18.5	13.58	28.03	13.77	14.17	0	0	0
4	Eagle Ray	*Halomonas*	3	0	20.96	9.23	30.4	15.57	13.3	0	0	0

5	Eagle Ray	*Halomonas*	1	17.94	24.78	7.56	28.16	6.49	9	0	0	0
5	Eagle Ray	*Halomonas*	2	11.71	15.71	15.19	33.75	5.74	9.04	0	0	0
5	Eagle Ray	*Halomonas*	3	1.54	0	0.66	28.15	5.23	8.96	0	0	0
6	Eagle Ray	*Pseudoalteromonas*	1	0.45	21.27	10.16	28.46	13.66	9.64	0	0	0
6	Eagle Ray	*Pseudoalteromonas*	2	10.48	21.14	15.57	25.27	14.01	12.4	0	0	0
6	Eagle Ray	*Pseudoalteromonas*	3	11.59	15.89	14.27	20.12	11.44	0	0	0	0
14	Eagle Ray	*Stutzerimonas*	1	0	10.76	0	8.95	15.78	13.93	0	0	0
14	Eagle Ray	*Stutzerimonas*	2	0	2.33	0	15.68	15.27	14.52	0	0	0
14	Eagle Ray	*Stutzerimonas*	3	0	2.06	0	9.26	24.52	14.87	0	0	0
ER 01	Eagle Ray	*Planococcus*	1	0.13	13.09	5.12	25.24	17.18	3.58	0	0	0
ER 01	Eagle Ray	*Planococcus*	2	0	15.26	6.53	24.71	18.45	8.11	0	0	0
ER 01	Eagle Ray	*Planococcus*	3	0.47	13.39	5.77	20.84	17.65	6.52	0	0	0
ER 02	Eagle Ray	*Marine bacterium*	1	3.55	39.23	1.9	33.55	23.04	14.44	0	0	0
ER 02	Eagle Ray	*Marine bacterium*	2	10.25	23.89	5.58	30.49	31.31	7.77	0	0	0
ER 02	Eagle Ray	*Marine bacterium*	3	11.57	35.62	5.6	23.81	36.88	28.57	0	0	0
ER 03	Eagle Ray	*Marine bacterium*	1	16.55	30.8	13.27	32.23	20.45	17.11	0	0	0
ER 03	Eagle Ray	*Marine bacterium*	2	18.5	34.25	13.53	34.25	22.47	18.15	0	0	0
ER 03	Eagle Ray	*Marine bacterium*	3	18.46	25.43	10.99	25.43	22.99	4.35	0	0	0
ER 04	Eagle Ray	*Pseudoalteromonas*	1	5.87	24.58	10.03	37.84	24.36	11.07	0	0	0
ER 04	Eagle Ray	*Pseudoalteromonas*	2	5.36	23.6	9.55	29.81	19.47	11.75	0	0	0
ER 04	Eagle Ray	*Pseudoalteromonas*	3	0.36	20.82	10.69	33.45	17.14	0	0	0	0
ER 05	Eagle Ray	*Marine bacterium*	1	1.9	23.47	8.52	15.97	15.24	2.87	0	0	0
ER 05	Eagle Ray	*Marine bacterium*	2	1.26	25.54	7.82	22.28	17.77	2.7	0	0	0
ER 05	Eagle Ray	*Marine bacterium*	3	10.13	29.6	6.68	27.73	21.46	7.76	0	0	0
ER 06	Eagle Ray	*Psychrobacter*	1	1.52	16.34	4.56	27.2	18.31	7.34	0	0	0
ER 06	Eagle Ray	*Psychrobacter*	2	3.88	17.25	6.66	27.01	15.91	7.44	0	0	0
ER 06	Eagle Ray	*Psychrobacter*	3	1.79	15.23	8.75	27.34	15.53	5.21	0	0	0
16	Fiddler Ray	*Marine bacterium*	1	1.24	22.34	8.87	28.33	16.41	7.07	0	0	0
16	Fiddler Ray	*Marine bacterium*	2	1.84	15.24	5.93	22.97	16.74	7.66	0	0	0
16	Fiddler Ray	*Marine bacterium*	3	0.79	14.03	8.64	19.57	16.91	10.11	0	0	0
17	Fiddler Ray	*Pseudoalteromonas*	1	1.23	14.98	8.63	26.58	11.51	3.91	0	0	0
17	Fiddler Ray	*Pseudoalteromonas*	2	0.45	11.03	8.05	23.85	15.28	3.29	0	0	0

17	Fiddler Ray	*Pseudoalteromonas*	3	0.78	12.71	7.99	22.94	13.41	9.79	0	0	0
18	Fiddler Ray	*Pseudoalteromonas*	1	5.74	19.96	9.78	22.27	18.41	4.87	0	0	0
18	Fiddler Ray	*Pseudoalteromonas*	2	8.57	18.32	10.41	25.53	20.34	6.52	0	0	0
18	Fiddler Ray	*Pseudoalteromonas*	3	5.05	22.63	11.11	18.72	19.18	1.28	0	0	0
19	Fiddler Ray	*Shewanella*	1	0	14.34	7.57	16.29	14.55	0	0	0	0
19	Fiddler Ray	*Shewanella*	2	0	14.84	4.85	20.96	12.22	6.42	0	0	0
19	Fiddler Ray	*Shewanella*	3	1.23	9.17	5.72	22.85	13.4	1.24	0	0	0
24	Fiddler Ray	*Pseudoalteromonas*	1	0	46.2	16.53	32.74	17.18	7.5	0	0	0
24	Fiddler Ray	*Pseudoalteromonas*	2	0	41.89	19.05	30.73	24.55	10.83	0	0	0
24	Fiddler Ray	*Pseudoalteromonas*	3	0	37.08	17.05	27.53	19.67	6.82	0	0	0
A05	Fiddler Ray	*Marine bacterium*	1	39.32	38.74	0	13.59	40.11	7.73	23.55	18.89	0
A05	Fiddler Ray	*Marine bacterium*	2	45.93	38.36	0	12.51	35.92	7.06	25.63	20.51	0
A05	Fiddler Ray	*Marine bacterium*	3	34.19	39.97	0	7.98	39.21	5.4	21.8	17.01	0
A06	Fiddler Ray	*Pseudoalteromonas*	1	24.34	31	0	14.47	31.83	6.39	18.09	8.41	0
A06	Fiddler Ray	*Pseudoalteromonas*	2	30.63	27.43	0	13.59	32.66	4.63	14.23	6.52	0
A06	Fiddler Ray	*Pseudoalteromonas*	3	29.61	27.42	0	11.04	35.7	8.35	11.34	5.75	0
A08	Fiddler Ray	*Vibrio*	1	15.56	15.38	3.75	3.4	12.54	0	0	0	0
A08	Fiddler Ray	*Vibrio*	2	11.26	22.7	0	22.06	15.78	0	0	0	0
A08	Fiddler Ray	*Vibrio*	3	11.1	14.51	4.3	14.57	13.64	0	0	0	0
A12	Fiddler Ray	*Pseudoalteromonas*	1	0	24.58	3.94	9.66	0	5.51	0	0	0
A12	Fiddler Ray	*Pseudoalteromonas*	2	0	24.6	3.07	9.88	0	5.76	0	0	0
A12	Fiddler Ray	*Pseudoalteromonas*	3	0	28.13	3.39	6.69	0	4.11	0	0	0
7	Melbourne Skate	*Stutzerimonas*	1	25.51	15.93	0	7.11	24.68	11.42	12.91	10.34	0
7	Melbourne Skate	*Stutzerimonas*	2	27.9	23.24	0	12.76	29.39	13.26	14.38	11.49	0
7	Melbourne Skate	*Stutzerimonas*	3	21.4	22.98	0	13.12	27.93	13.84	20.41	10.32	0
9	Melbourne Skate	*Marine bacterium*	1	8.58	23.26	4.57	10.63	16.13	2.71	0	0	0
9	Melbourne Skate	*Marine bacterium*	2	9.34	15.59	4.54	7.81	10.67	2.76	0	0	0
9	Melbourne Skate	*Marine bacterium*	3	6.64	13.79	8.08	6.89	11.52	0.77	0	0	0
10	Melbourne Skate	*Pseudoalteromonas*	1	0	11.19	0	7	0	3.44	0	0	0
10	Melbourne Skate	*Pseudoalteromonas*	2	0	14.13	0	7.43	0	4.87	0	0	0
10	Melbourne Skate	*Pseudoalteromonas*	3	0	17.59	0	8.83	0	5.4	0	0	0
11	Melbourne Skate	*Marine bacterium*	1	7.7	10.28	8.67	6.18	8.52	1.5	0	0	0

11	Melbourn e Skate	*Marine bacterium*	2	6.66	11.7	5.86	6.95	5.74	2.79	0	0	0
11	Melbourn e Skate	*Marine bacterium*	3	4.74	10.79	5.64	10.76	6.44	1.99	0	0	0
12	Melbourn e Skate	*Pseudoalte romonas*	1	0	17.71	0	24.51	13.9	7.44	0	0	0
12	Melbourn e Skate	*Pseudoalte romonas*	2	0	15.03	0	24.64	13.91	6.85	0	0	0
12	Melbourn e Skate	*Pseudoalte romonas*	3	0	19.53	0	26.29	12.32	4.45	0	0	0
13	Melbourn e Skate	*Pseudoalte romonas*	1	7.49	23.12	5.89	4.19	25.62	4.62	7.24	0	0
13	Melbourn e Skate	*Pseudoalte romonas*	2	4.67	19.54	8.33	2.27	21.46	4.4	7.9	0	0
13	Melbourn e Skate	*Pseudoalte romonas*	3	2.96	17.61	6.81	1.14	28.13	3.59	8.61	0	0
15	Melbourn e Skate	*Pseudoalte romonas*	1	3.16	22	2.44	6.82	18.05	10.97	0	0	0
15	Melbourn e Skate	*Pseudoalte romonas*	2	12.76	15.53	5.07	2.24	19	6.35	0	0	0
15	Melbourn e Skate	*Pseudoalte romonas*	3	14.41	18.59	3.94	5.2	15.29	8.66	0	0	0
21	Port Jackson Shark	*Pseudoalte romonas*	1	8.61	19.5	4.66	18.95	12	9.2	0	0	0
21	Port Jackson Shark	*Pseudoalte romonas*	2	4.04	17.9	7.75	20.04	13.17	10.85	0	0	0
21	Port Jackson Shark	*Pseudoalte romonas*	3	11.64	15.09	10.99	13.14	14.38	5.97	0	0	0
22	Port Jackson Shark	*Pseudoalte romonas*	1	15.23	18.52	6.46	16.62	18.44	0	0	0	0
22	Port Jackson Shark	*Pseudoalte romonas*	2	13.26	21.24	5.7	17.81	18.24	0	0	0	0
22	Port Jackson Shark	*Pseudoalte romonas*	3	13.8	18.91	5.75	16.05	15.69	0	0	0	0
23	Port Jackson Shark	*Pseudoalte romonas*	1	9.04	16.18	3.45	2.05	15.2	6.22	0	0	0
23	Port Jackson Shark	*Pseudoalte romonas*	2	1.75	17.62	6.51	4.9	18.55	6.32	0	0	0
23	Port Jackson Shark	*Pseudoalte romonas*	3	2.3	16.31	0	3.8	12.44	10.51	0	0	0
25	Sevegills Shark	*Pseudoalte romonas*	1	4.97	18.78	0	4.42	9.39	4.04	0	0	0
25	Sevegills Shark	*Pseudoalte romonas*	2	1.7	21.04	0	1.52	15.69	4.92	0	0	0
25	Sevegills Shark	*Pseudoalte romonas*	3	2.29	17.06	0	5.95	17.71	5.29	0	0	0

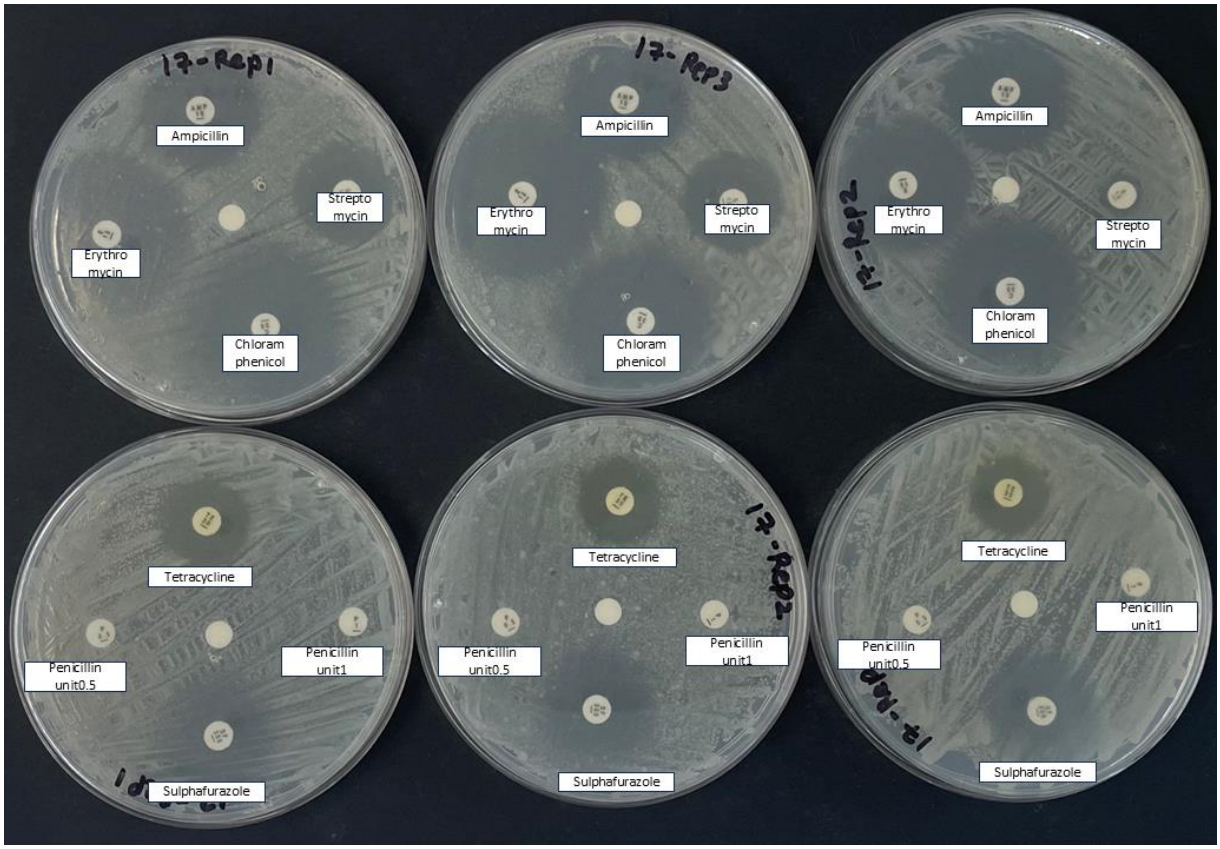

Figure 12. Photo of antibiotic susceptibility test (disc diffusion). This image shows a set of M2216 marine agar plates used to test the sensitivity of *Pseudoalteromonas* sp., isolated from the skin microbiome of the fiddler ray, to eight different antibiotics. The antibiotics tested include Ampicillin, Erythromycin, Streptomycin, Chloramphenicol, Tetracycline, Penicillin (0.5 units and 1 unit), and Sulfafurazole. Each plate represents one of three replicates performed during the experiment. The central blank sterile disc on each plate serves as a negative control, consistently showing 0 mm of zone of inhibition.

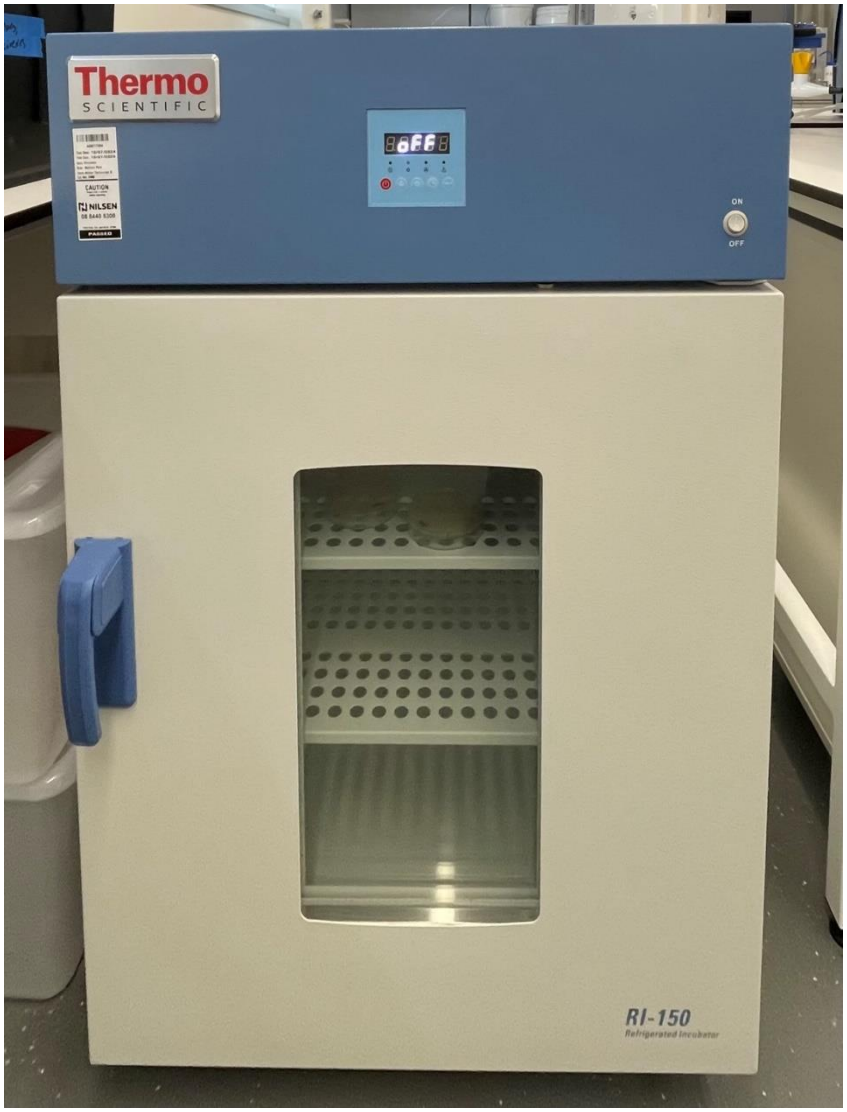

Figure 13. ThermoFisher Scientific RI-150. This refrigerated incubator used for growing isolated bacteria from elasmobranchs. This incubator maintained a constant temperature of 18°C, ensuring optimal conditions for marine microbial growth during the disc diffusion assays for antibiotic sensitivity testing.

Figure 14. Eppendorf Mastercycler Nexus Gradient PCR machine. it used for the amplification of DNA from the skin microbiome of elasmobranchs. This instrument was essential for both PCR amplification and cleaning PCR products, offering flexibility in setting temperature gradients and time periods for each stage of the PCR cycle, ensuring accurate and reliable results during genetic analysis of the isolated microbes.