UNIVERSIDADE FEDERAL DO PARANÁ

ANDRÉIA BUENO DA SILVA

BIOPROSPECÇÂO DE BACTÉRIAS DE ORIGEM AMBIENTAL E CLÍNICAS DAS COLEÇÕES MICROBIOLÓGICAS DA REDE PARANAENSE (CMRP-TAXONLINE)

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2024

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Tese apresentada para defesa de doutorado ao Programa de Pós-Graduação em Microbiologia, Parasitologia e Patologia, Setor de Ciências Biológicas, da Universidade Federal do Paraná, como requisito parcial para obtenção do título de Doutora em Microbiologia, Parasitologia e Patologia.

Orientadora: Prof.ª Dra. Vania Aparecida Vicente

Coorientadora: Prof.ª Dra. Keite da Silva Nogueira

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I dedicate this work to God,
who sustained me at every stage of my life.
To my family and friends.
And for Brazilian Science.

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"Sometimes we feel that what we do is just a drop in the ocean.
But the sea would be smaller if it lacked a drop" (Madre Teresa de Calcuta).

RESUMO

As bactérias multirresistentes (MDR) representa um desafio significativo para a saúde global, pois esses patógenos tornam ineficazes os antibióticos disponíveis e contribuem para o aumento das taxas de morbidade e mortalidade. Nesse contexto, o presente estudo examinou os perfis multirresistentes de sessenta e cinco isolados clínicos obtidos de um hospital universitário em Curitiba, Brasil, entre 2011 e 2021. O objetivo foi caracterizar as linhagens com perfil de resistência por meio da implementação de ensaios microbiológicos e moleculares, bem como da identificação das espécies por MALDI-TOF. De acordo com a definição fornecida pela Organização Mundial da Saúde (OMS), essas bactérias multirresistentes são identificadas como agentes causais prioritários que devem ser identificados e depositados em centros de coleta de referência. Para facilitar pesquisas futuras, as linhagens caracterizadas neste estudo foram depositadas no Centro de Coleções Microbiológicas da Rede Taxonline do Paraná (CMRP/Taxonline, https://www.cmrp-taxonline.com/). As bactérias patogênicas multirresistentes estudadas mostraram-se valiosas para pesquisas futuras, ressaltando a importância disso para a implementação de estratégias personalizadas e programas de administração de antibióticos contra bactérias multirresistentes. Além disso, o estudo examinou cepas de *Streptomyces* spp. isoladas de sedimentos marinhos na Ilha do Mel, Paraná, Brasil, que foram previamente identificadas como tendo potencial atividade antimicrobiana. Os isolados ambientais foram examinados por meio de testes de suscetibilidade *in vitro*, que demonstraram atividade antimicrobiana contra bactérias multirresistentes, incluindo cepas patogênicas Gram-positivas e Gram-negativas com vários mecanismos de resistência. No entanto, a espécie *Streptomyces cavourensis* CMRP6046 demonstrou um potencial antimicrobiano considerável. O genoma dessa espécie foi sequenciado em sua totalidade, o que levou à identificação de genes que supostamente codificam possíveis compostos antibacterianos. A análise revelou um número substancial de genes envolvidos na biossíntese de metabólitos secundários, indicando o potencial da *S. cavourensis* para a produção de novos compostos. Essas descobertas ressaltam a importância do *Streptomyces* spp. na busca de novos compostos antimicrobianos e destacam a necessidade de explorar a biodiversidade microbiana para aplicações biotecnológicas. Outras pesquisas devem se concentrar na ativação de genes biossintéticos e na utilização de perfis metabólicos para o desenvolvimento de estratégias inovadoras de combate à resistência antimicrobiana.

Palavras-chave: *Streptomyces*; resistência bacteriana; genoma completo; metabólitos secundários; clusters gênicos biossintéticos.

ABSTRACT

The emergence of multidrug resistant (MDR) bacteria represents a significant challenge for global health, as these pathogens render available antibiotics ineffective and contribute to increased morbidity and mortality rates. In this context, the present study examined the multidrug resistant profiles of sixty-five clinical isolates obtained from a university hospital in Curitiba, Brazil, between 2011 and 2021. The objective was to characterize the strains with a resistance profile through the implementation of microbiological and molecular assays, as well as species identification by MALDI-TOF. In accordance with the definition provided by the World Health Organization (WHO), these multidrug resistant (MDR) bacteria are identified as priority causal agents that should be identified and deposited in reference collection centers. To facilitate future research, the strains characterized in this study have been deposited in the Center for Microbiological Collections of the Paraná Taxonline Network (CMRP/Taxonline, https://www.cmrp-taxonline.com/). The multidrug resistant pathogenic bacteria studied proved valuable for future research, underscoring the significance of this for the implementation of personalized strategies and antibiotic stewardship programs against multidrug resistant bacteria. Furthermore, the study examined *Streptomyces* spp. strains isolated from marine sediments on Ilha do Mel, Paraná, Brazil, which have previously been identified as having potential antimicrobial activity. Environmental isolates were examined using *in vitro* susceptibility tests, which demonstrated significant antimicrobial activity against multidrug resistant bacteria, including Gram-positive and Gram-negative pathogenic strains with various resistance mechanisms. Nevertheless, the *Streptomyces cavourensis* CMRP6046 species demonstrated considerable antimicrobial potential. The genome of this species was sequenced in its entirety, which led to the identification of genes that are thought to encode potential antibacterial compounds. The analysis revealed a substantial number of genes involved in the biosynthesis of secondary metabolites, indicating the potential of *S. cavourensis* to produce new compounds. These findings underscore the importance of *Streptomyces* spp. in the search for new antimicrobial compounds and highlight the necessity of exploiting microbial biodiversity for biotechnological applications. Further research should concentrate on the activation of biosynthetic genes and the utilization of metabolic profiles for the development of innovative strategies to combat antimicrobial resistance.

Keywords: *Streptomyces*; antimicrobial resistance; whole genome; secondary metabolites; biosynthetic gene clusters.

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LIST OF ABBREVIATIONS OR ACRONYMS

ATCC	- American Type Culture Collection
BGC	- Biosynthetic gene cluster
CBM	- Minimum bactericidal concentration
CFF	- Cell-free filtrate
CFU	- Colony forming unit
CIA	- Chloroform: isoamyl alcohol
CLSI	- Clinical and Laboratory Standards Institute
CTAB	- Cetyltrimethylammonium bromide
DMSO	- Dimethyl sulfoxide
ESBL	- Extended Spectrum Betalactamases
ESKAPEE	-*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species
EUCAST	- The European Committee on Antimicrobial Susceptibility Testing
GY	- Glucose Yeast Extract
MDR	- Multidrug resistant
MHB	- Mueller-Hinton Broth
MIC	- Minimum Inhibitory Concentration
MRSA	- Methicillin Resistant *Staphylococcus aureus*
PCR	- Polymerase Chain Reaction
TSB	- Trypticase Soy Broth
VRE	- Vancomycin Resistant *Enterococcus*
WHO	- World Health Organization

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1 GENERAL INTRODUCTION

Environmental microorganisms play a crucial role in the synthesis and production of a wide range of natural products (Abdel-Razek et al., 202). They are capable of synthesizing compounds with an impressive variety of applications, including antibiotics, antitumor agents, antioxidants, industrial enzymes, and many other products of pharmaceutical and industrial interest (Alam et al; Kaari et al., 2022; Ghattavi; Homaei, 2023).

Environmental microorganisms include bacteria, which have a remarkable ability to produce compounds with antimicrobial activity. This ability is an evolutionary strategy to compete for resources and survive in their natural habitats (Kandasamy and Kathirvel, 2023). Several studies have shown that bacteria isolated from different environments such as soils, seawater, sediments, and plants are rich sources of metabolites with antimicrobial properties (Jagannathan et al., 2021; Wen et al., 2022; Sharma et al., 2023).

Given this, bacterial isolates from various environments have been an important source in discovering and developing new antibiotics, especially in the face of the growing challenge of bacterial resistance. A group of bacteria known as "ESKAPEE" - *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp. and *Escherichia coli* - are of particular concern due to their ability to "escape" the action of multiple antibiotics (Murugaiyan et al., 2022).

In 2024, the WHO published a list of "priority and high-priority pathogens" resistant to antibiotics, developed to guide research and development efforts for new antibiotics and to emphasize the importance of controlling antimicrobial resistance on a global scale. The most critical group includes multidrug resistant bacteria, which pose a particular threat in hospitals, nursing homes and patients whose care requires devices such as ventilators and blood catheters (WHO, 2024).

Based on it, the present work reports on the study of four marine sediment isolates of the genus *Streptomyces* against twelve antibiotic-resistant bacterial clinical strains from the Hospital de Clinica/UFPR, Paraná for obtain new bioactive compounds with antibacterial potential. In addition, genomic sequencing was performed on the most promising strain of *Streptomyces cavourensis* CMRP6046. All strains are

deposited in the Microbiological Collections Centre of the Taxonline Network of Paraná (CMRP/Taxonline).

This thesis is structured in four chapters to organize the different topics covered. In Chapter I, a comprehensive review of the antimicrobial potential of bacteria isolated from different environments is carried out. Chapter II identifies and characterizes bacteria and resistance profiles using molecular techniques. Chapter III explores the antimicrobial potential of species belonging to the bacterial genus *Streptomyces*, isolated from the marine sediment of Ilha do Mel, Paraná, in confrontation with multidrug resistant clinical bacteria. Finally, Chapter IV describes the complete genome of the *Streptomyces cavourensis* strain CMRP6046 and the gene clusters responsible for producing secondary metabolites.

1.1 OBJECTIVES

1.1.1 General Objectives

Bioprospect environmental bacteria of biotechnological interest against multidrug resistant clinical bacteria deposited at the Microbiological Collections Center of the Paraná Taxonline Network (CMRP/Taxonline).

1.1.2 Specific objectives

- Review the antimicrobial potential of bacteria of environmental origin;
- Characterize the multidrug resistance profile of clinical bacterial strains using molecular techniques;
- Identify the antimicrobial activity of the alcoholic extract of *Streptomyces* sp isolated from the marine environment against multi-resistant bacteria;
- Use bioinformatics tools to identify the genes in the genome of *Streptomyces cavourensis* CMRP6046 responsible to produce secondary metabolites for biotechnological application.

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2 CHAPTER I

ENVIRONMENTAL BACTERIAL SPECIES WITH ANTIMICROBIAL POTENTIAL AGAINST ESKAPEE PATHOGENS: A SCOPING REVIEW

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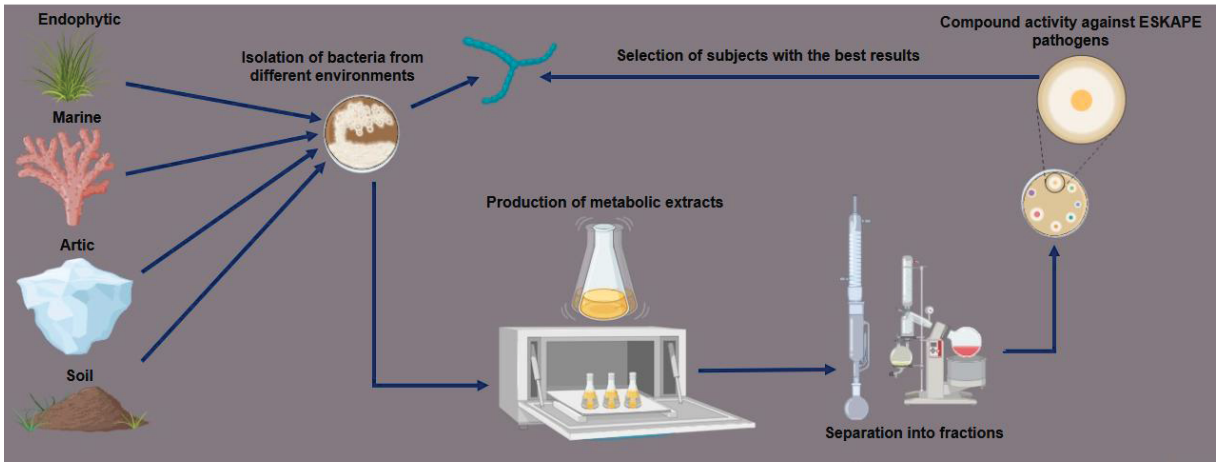
FIGURE 1 - GRAPHICAL ABSTRACT



SOURCE: The author (2024).

2.1 ABSTRACT

Antimicrobial resistance poses a serious threat to global health, complicating the treatment of infectious diseases and increasing mortality rates and healthcare expenditures. Although antibiotic use in some areas has decreased, multidrug resistant bacteria, especially those among significant pathogens such as ESKAPEE, are a major global challenge. The aim of this review was to gather data on bacterial genera from various environmental sources that synthesize compounds with antimicrobial activity against pathogens belonging to the ESKAPEE group. The MEDLINE/PubMed, Scopus, and Science Direct databases were searched for articles published from 2001

to 2023. The included publications were carefully selected based on their relevance to the topic and methodologies used. In total, 50 articles were reviewed and 20 bacterial genera with significant antimicrobial activity were identified, with Streptomyces spp. being the most prevalent. The bacteria were isolated from different environments, including marine sediments, endophytes, and extreme environments. The evaluation of bacterial extracts against ESKAPEE pathogens revealed considerable inhibition capacities, particularly for *Streptomyces* spp., *Bacillus* spp. and *Pseudomonas* spp. However, no single extract was effective in inhibiting all target pathogens. Furthermore, 33 bacterial extracts from the 50 studies included in the review characterized, at least partially, the secondary metabolites responsible for the observed antibacterial potential. Twenty patents filed for compounds characterized with antibacterial activity against ESKAPEE bacteria were found. And in the search for marketed products, a total of 22 compounds were found. The discussion highlights the marine environment as a rich source of antimicrobial-producing bacteria due to its unique ecological conditions. *Streptomyces* spp. remain promising candidates for antibiotic discovery, due to a diverse range of antimicrobial compounds. In conclusion, this review highlights the strong ability of bacteria from a wide range of environments to combat antimicrobial resistance. By elucidating antimicrobial activity and compound characterization, this review provides valuable insights into antibiotic research, crucial to managing the growing threat of multidrug resistant pathogens.

Keywords: multidrug resistance; bioactive compounds; *Streptomyces*; marine sediment.

2.2 INTRODUCTION

Antimicrobial resistance (AMR) greatly threatens global health (Jee et al., 2018; Murray et al., 2022) which has made it difficult to treat infectious diseases and, consequently, increased mortality rates (Murugaiyan et al., 2022). The increase in AMR, particularly among clinically important ESKAPEE pathogens (*Enterococcus* species, *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *Escherichia coli*), has greatly burdened the healthcare system and the veterinary and agricultural industries (Ruekit et al., 2022). By 2050, 10 million deaths are estimated to occur per year due to AMR caused by ESKAPEE pathogens, resulting in a cumulative cost of $100 trillion to the global economy if no preventive action is taken (O'Neill, J., 2016; Poudel et al., 2023).

According to the World Health Organization (WHO), 30 new antibiotics were approved for treating bacterial infection; however, only two were new compounds (Efimenko et al., 2018). Thus, new antibiotics with new compounds need to be developed to reduce the effect of AMR, as multidrug resistant (MDR) strains can be resistant to most antibiotic classes (WHO, 2021; WHO, 2024).

Microorganisms inhabiting a variety of environments, especially extreme and unexplored environments, are a promising source of novel antimicrobial compounds (Quinn and Dyson, 2024). Among the microorganisms that produce bioactive compounds, bacteria are prominent for their ability to produce various secondary metabolites with antimicrobial activity (Srinivasan et al., 2021; Barzkar et al., 2021).

In this scenario, this review addresses relevant publications on bioprospecting of bacterial strains isolated from diverse environmental sources as a promising strategy to identify compounds with bioactivities against the ESKAPEE group. Additionally, the characterized chemical compounds, the applied analytical methods and an analysis of registered patent data were discussed.

2.3 MATERIALS AND METHODS

This scoping review was conducted following the guidelines of the Transparent Reporting of Systematic Reviews and Meta-Analyses for Scoping Reviews (PRISMA-ScR) extension (Tricco et al., 2018) to address the following question, according to the context, concept, and population (CCP) criteria/guidelines: "*What is the antimicrobial potential of bacteria of environmental origin against multidrug resistant bacteria*?" Three online databases were searched for suitable articles published from 2001 to 2023 that met the aim of this review: MEDLINE (via PubMed), Scopus (Elsevier: Amsterdam, Netherlands), and Science Direct (Elsevier: Anglo-Dutch).

Peer-reviewed studies describing the production of bacterial extracts with antibiotic activity against MDR ESKAPEE pathogens were included in the review. Articles were searched using the following keywords: "antibacterial activity"; "secondary metabolites*"; "bioactive compounds" and "multidrug resistant bacteria". The keywords were combined with the Boolean operators "AND" or "OR" with the proximity operators ["" and ()] and the truncation operator (*) when required. The search strategy was modified according to the databases (Figure 1). The electronic database search was supplemented by a manual search using references from all included articles. As search criteria included information on the country of isolation, the environmental source of strain, the taxonomic status from at least the genus level, the deposit code for the culture collection, the ESKAPEE pathogen inhibition capacity, the compound characterization (if available), and the extraction fraction used for inhibition. The studies that did not include the information described above and those that did not

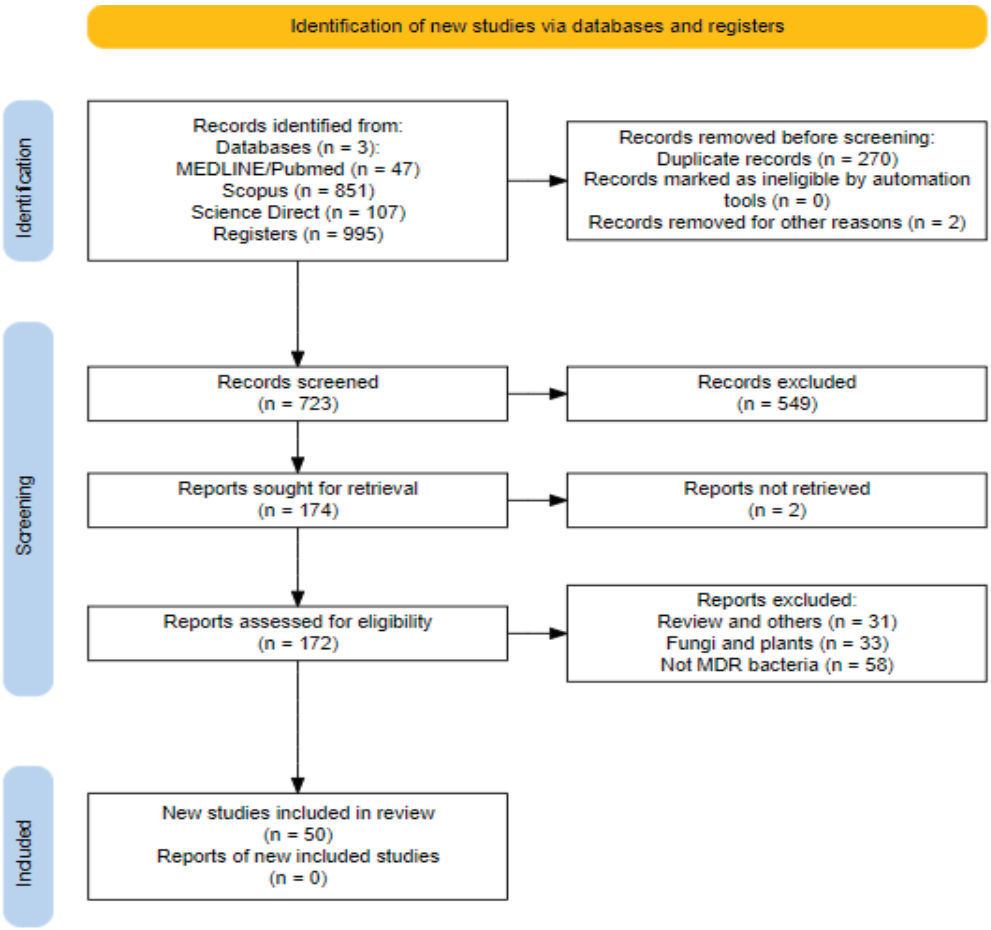
evaluate antimicrobial activity against AMR pathogens were excluded. The type of article included was also restricted; reviews, short communications, conference abstracts, preprints, and book chapters were excluded.

Patent databases were searched to identify emerging biotechnological trends in the field of chemical compounds with antibacterial activity. The patent numbers were collated using the advanced search option (title or abstract) from the following databases: the Espacenet patent search (worldwide.espacenet.com), the INPI (National Institute of Intellectual Property) Intellectual Patent (https:www.gov.br/inpi/en-br), and the PubChem (https://pubchem.ncbi.nlm.nih.gov/) databases. The analysis of patents was limited to those published between 2010 and 2023. In the search bar of the respective websites, the terms "antibacterial" or "antibiotic" were entered along with the name of the compound described in the article.

2.4 RESULTS

The initial search resulted in 995 studies, of which 47 were in MEDLINE/PubMed, 841 in Scopus, and 107 in Science Direct. After removing 270 duplicate studies, 549 studies were rejected after screening the articles by their titles and abstracts. The remaining 174 studies were retrieved and analyzed. Overall, 50 articles published from 2001 to 2023 met the inclusion criteria. The flowchart of the search strategy is shown in Figure 2.

FIGURE 2 - PRISMA FLOWCHART FOR SEARCH STRATEGIES

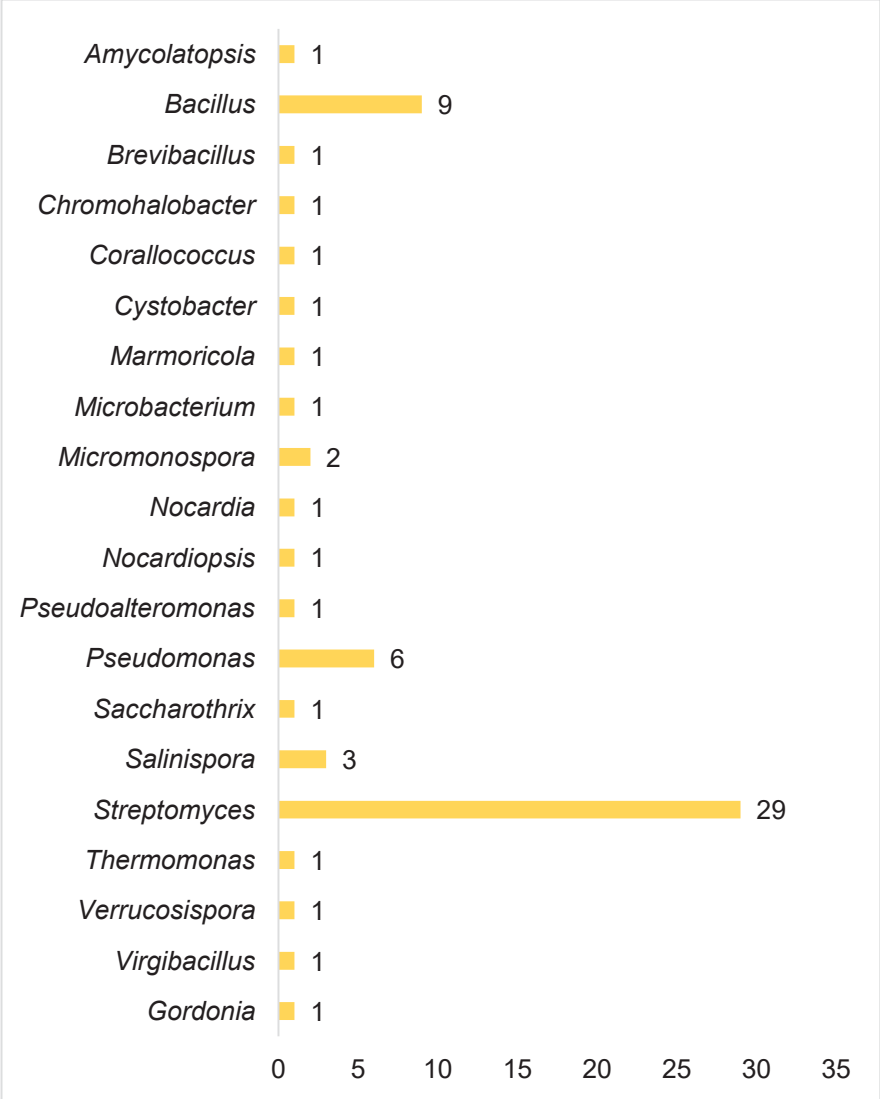


Notes: Flowchart structured using R Program (Haddaway et al., 2022).

2.4.1 Taxonomic and isolation source of environmental bacteria with antimicrobial potential.

From the studies selected, 20 bacterial genera capable of producing bioactive compounds were identified (Figure 2). The most frequent genus with antimicrobial production potential was *Streptomyces* spp. (n = 27), followed by *Bacillus* spp. (n = 8), *Pseudomonas* spp. (n = 5), and *Salinispora* sp. (n = 3) (Figure 3).

FIGURE 3 - NUMBER OF ISOLATES BY GENERA (N=64) BASED ON THE FIFTY PUBLICATIONS GATHERED



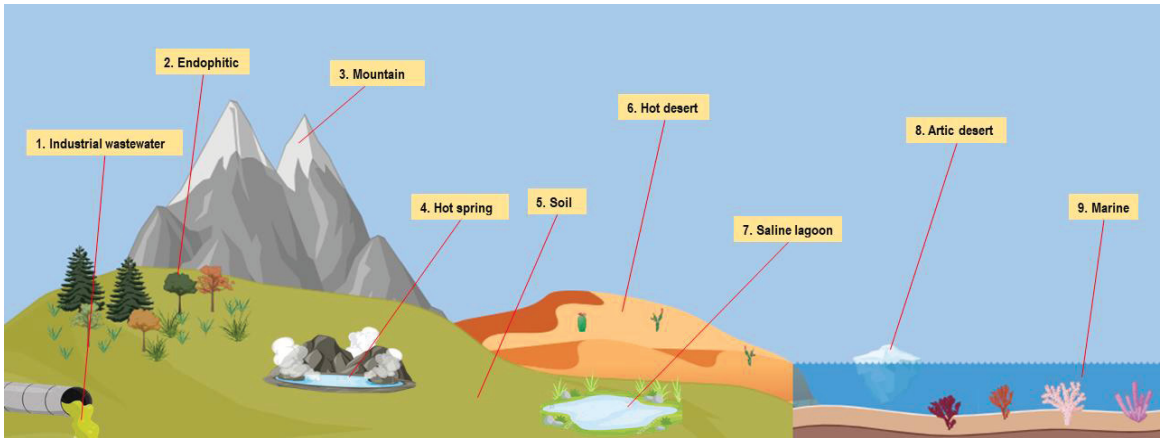
SOURCE: The author (2024).

Notes: Some publications have more than one isolate; therefore, it is computed according to the total of isolates tested in the study.

The studies reported species isolated from various extreme environments, such as hot springs (n = 1), ice deserts (n = 3), deserts (n = 5), saline lagoons (n = 1), and soil from high-altitude mountains (n = 2), as described in Figure 3. The isolates were also found in marine sediments (n = 4) associated or not associated with animals such as sea sponges (n = 6) and sea slugs (n = 2) and associated with plants as endophytes (n = 6). The most diverse sources were endophytes and deserts (both n = 6). Some species were isolated from a single source; for example, *Thermomonas* sp. was the only species isolated from hot springs, whereas the genus *Streptomyces*

was the most diverse and was found in seven different environments; it was also the only genus isolated from saline lagoons (Figure 4).

FIGURE 4 - SCHEMATIC PICTURE OF THE MAIN SOURCES OF ISOLATION OF BACTERIA WITH POTENTIAL TO PRODUCE ANTIBIOTICS COMPOUNDS TO TREAT ESKAPEE PATHOGENS



SOURCE: The author (2024).

Streptomyces spp. was the most common genus isolated from endophytic, industrial wastewater, and marine environments that might represent an important source of antimicrobial compounds (Table 1). This genus was isolated from endophytic plants in Brazil, China, India, Indonesia, and Malaysia, industrial wastewater from India, marine sediments from Argelia, Costa Rica, India, Morocco, Peru, Philippines, and Saudi Arabia, mountains from China, saline lagoons from Peru, sea sponges from India, and soils from Iran, Ireland, Nigeria, and Thailand. Moreover, it was observed other genera able to produce bioactive compounds such as *Bacillus* spp. isolated from plants in Indonesia, marine algae and sea sponges in India, and extreme environments such as ice deserts in Antarctica. Likewise, *Pseudomonas* spp. isolated as endophytes from the medicinal plant Phragmites *australis* in Italy, from sea sponges in Brazil, India, and Malaysia and from ice deserts in the Canadian tundra (Table 1).

TABLE 1 - MAIN ENVIRONMENTAL SOURCES ARE INDICATED IN THE BOXES AS SPECIFIED BY THE GENERA FROM THE STRAINS ISOLATED

Number*	Environmental sources	Bacterial genera
1	Industrial Wastewater	*Streptomyces* spp. *Bacillus* spp.
2	Endophytic	*Bacillus* spp. *Brevibacillus* spp. *Marmoricola* spp. *Pseudomonas* spp. *Streptomyces* spp. *Verrucosispora* spp
3	Mountain High altitude	*Amycolatopsis* spp. *Streptomyces* spp.
4	Hot spring	*Thermomonas* spp.
5	Soil	*Serratia* spp. *Streptomyces* spp.
6	Hot desert	*Corallococcus* spp. *Cystobacter* spp. *Microbacterium* spp. *Nocardia* spp. *Saccharothrix* spp.
7	Saline Lagoon	*Streptomyces* spp.
8	Artic desert	*Bacillus* spp. *Gordonia* spp. *Pseudomonas* spp.
9	Marine (symbiote Marine algae)	*Bacillus* spp.
9	Marine	*Micromonospora* spp. *Nocardiopsis* spp. *Salinispora* spp. *Streptomyces* spp.
9	Marine (symbiote Sea Slug)	*Pseudoalteromonas* spp. *Virgibacillus* spp.
9	Marine (symbiote Sea Sponge)	*Bacillus* spp. *Chromohalobacter* spp. *Micromonospora* spp. *Pseudomonas* spp. *Salinispora* spp. *Streptomyces* spp.

SOURCE: The author (2024).

Notes: *Number is according to the diagram in figure 4

Among the most cryptic bacteria, *Salinispora* spp. were isolated from marine sediment in Mexico and from sea sponges in Fiji (Singh et al., 2014; Contreras-Castro et al., 2020). *Amycolatopsis* sp. was isolated solely from high mountains in China (Qian et al., 2022). *Brevibacillus brevis* was isolated from an endophytic environment in India (Arumugam et al., 2017), *Chromohalobacter salixipes* was isolated from a sea sponge in Indonesia (Asagabaldan et al., 2017), *Corallococcus* sp. and *Cystobacter* sp. were isolated from deserts in Iran (Saadatpour et al., 2020), *Gordonia terrae* was isolated from an ice desert in the Antarctic (Efimenko et al., 2018), *Marmoricola* sp. was isolated from plants in China (Jiang et al., 2018), *Microbacterium* sp. was isolated from a desert

in China (Liu et al., 2021), *Micromonospora marina* was isolated from marine sediment in India (Raja et al., 2023), *Micromonospora robiginosa* was isolated from a sea sponge in the UK (Back et al., 2021), *Nocardia* sp. was isolated from a desert in Iran (Hamedi et al., 2015), *Nocardiopsis* sp. was isolated from marine sediment in Switzerland (Engelhardt et al., 2010), *Pseudoalteromonas rubra* and *Virgibacillus salaries* were isolated from sea slugs in Indonesia (Kristiana et al., 2020), *Saccharothrix* sp. was isolated from a desert in China (Liu et al., 2021), *Serratia marcescens* was isolated from soil in India (Arivuselvam et al., 2023), *Thermomonas hydrothermalis* was isolated from a hot spring in Jordan (Al-Daghistani et al., 2021), and *Verrucosispora* sp. was isolated from plants in Brazil (Assad et al., 2021) (Table 1).

2.4.2 Antibacterial activity of the environmental strains against ESKAPEE pathogens

According to the records of this review (Figure 1), 64 isolates were tested against ESKAPEE pathogens (details in Table 2). The most common genus tested was *Streptomyces*, which can inhibit methicillin-resistant and MDR *Staphylococcus aureus* (MRSA and MDRSA), vancomycin-resistant and MDR *Enterococcus* (VRE and MDRE), MDR *Pseudomonas* (MDRP), MDR *Acinetobacter baumannii* (MDRA), MDR *Klebsiella* (MDRK), and MDR *Escherichia coli* (MDREc). Additionally, the extract from *Bacillus* can inhibit MRSA, MDRSA, VRE, MDRK, and MDREc, whereas the extract from *Pseudomonas* can inhibit MRSA, MDRSA, MDRK, and MDREc. However, no extract tested could inhibit all the ESKAPEE pathogens simultaneously (Table 2).

Only four genera (*Chromohalobacter*, *Micrococcus*, *Saccharothrix,* and *Streptomyces*) showed activity against MDRA, and three genera (*Bacillus*, *Pseudomonas,* and *Streptomyces*) showed activity against MDREc. Moreover, *Streptomyces* exhibited the strongest inhibitory effects on ESKAPEE pathogens, with three isolates demonstrating the ability to inhibit six distinct pathogens, including MDRA, MDRK, and MDRSA (Table 2).

The studies showed that when crossing environments with antibacterial activity, all strains isolated from deserts (Hamedi et al., 2015; Saadatpour et al., 2020; Liu et al., 2021) and ice deserts (Efimenko et al., 2018; Marcolefas et al., 2019) exhibited activity against MRSA (Table 2). Additionally, the isolation of actinobacteria from marine environments (marine sediment, sea sponges, sea slugs, and marine algae) revealed that this environment had the greatest capacity to inhibit all ESKAPEE

pathogens (Table 3). The strains isolated from marine sediments, including *Streptomyces rochei* PM49 (Shanthi et al., 2015) and *Streptomyces* sp. Al-Dhabi-90 (Al-Dhabi et al., 2019), showed the greatest activity against MDR bacteria; their alcoholic extracts can inhibit the growth of six different MDR bacteria (Table 2).

TABLE 2 - ANTIBACTERIAL ACTIVITY OF ENVIRONMENTAL ISOLATES AGAINST ESKAPEE PATHOGENS, ACCORDING TO THE COUNTRY OF ORIGIN, SOURCE, AND EXTRACT (Continue)

Specie | Strain	Country	Source	Extract	Antibacterial activity								Reference
				MRSA	MDS	VRE	MDRE	MDRP	MDRA	MDK	MDREc	
Amycolatopsis sp. | YNNP 00208	China	Mountain (High altitude)	alcoholic	-	+	-	-	-	-	-	-	Qian et al., 2022
Bacillus amyloliquefaciens | MTCC 12713	India	Marine algae	alcoholic	+	-	+	-	-	-	-	-	Chakraborty et al., 2022
Bacillus amyloliquefaciens | MTCC 12716	India	Marine algae	alcoholic	+	-	+	-	-	-	-	-	Chakraborty et al., 2021
Bacillus licheniformis | INA 01155	Antarctic	Ice desert (Artic)	water	+	-	-	-	-	-	-	-	Efimenko et al., 2018
Bacillus safensis | INA 01154	Antarctic	Ice desert (Artic)	water	+	-	-	-	+	-	-	-	Efimenko et al., 2018
Bacillus subtilis| TAAAP010	India	Industrial wastewater	alcoholic	-	-	-	+	+	-	-	-	Kumar et al., 2021
Bacillus sp. | DJ4	Indonesia	Endophytic	alcoholic	-	-	-	-	+	-	+	+	Priyanto et al., 2023
Bacillus sp. | DJ9	Indonesia	Endophytic	alcoholic	-	-	-	-	+	-	+	+	Priyanto et al., 2023
Bacillus tequilensis | MSI45	India	Marine Sponge	alcoholic	+	+	-	-	-	-	-	-	Kiran et al., 2018
Bacillus velezensis | MTCC13048	India	Marine algae	alcoholic	+	-	-	-	-	-	-	-	Chakraborty et al., 2021
Brevibacillus brevis | EGS9	India	Endophytic	alcoholic	+	+	-	-	-	-	-	-	Arumugam et al., 2017
Chromohalobacter salixigens | PSP. 39-04	Indonesia	Sea Sponge	alcoholic	+	-	+	+	+	+	-	-	Asagabaldan et al., 2017
Corallococcus sp. | UTMC 4088	Iran	Hot desert	alcoholic	+	-	-	-	-	-	-	-	Saadatpour and Mohammadipanah, 2020
Cystobacter sp. | UTMC 4086	Iran	Hot desert	alcoholic	+	-	-	-	-	-	-	-	Saadatpour and Mohammadipanah, 2020
Gordonia terrae | INA 01165	Antarctic	Ice desert (Artic)	water	+	-	-	-	-	-	-	-	Efimenko et al., 2018
Marmoricola sp. | 8BTY-J2	China	Endophytic	alcoholic	+	+	-	-	-	+	-	-	Jiang et al., 2018

TABLE 2 - ANTIBACTERIAL ACTIVITY OF ENVIRONMENTAL ISOLATES AGAINST ESKAPEE PATHOGENS, ACCORDING TO THE COUNTRY OF ORIGIN, SOURCE, AND EXTRACT (Continuation)

Specie | Strain	Country	Source	Extract	Antibacterial activity								Reference
				MRSA	MDS	VRE	MDRE	MDRP	MDRA	MDK	MDREc	
Microbacterium sp. | 16Sb5-9	China	Hot desert	alcoholic	+	-	-	-	-	-	-	-	Liu et al., 2021
Micromonospora marina | KPMS1	India	Marine Sediment	alcoholic	-	-	-	-	-	-	+	-	Raja et al., 2023
Micromonospora robiginosa | 28ISP2-46	UK	Sea Sponge	alcoholic	+	+	-	-	-	-	-	-	Back et al., 2021
Nocardia sp. | UTMC 751	Iran	Hot desert	alcoholic	+	-	-	-	-	-	-	-	Hamedi et al., 2015
Nocardiopsis sp. | TFS65-07	Switzerland	Marine Sediment	alcoholic	-	-	+	-	-	-	-	-	Engelhardt et al., 2010
Pseudoalteromonas rubra	Indonesia	Sea slug	alcoholic	+	-	-	-	-	-	-	-	Kristiana et al., 2020
Pseudomonas aeruginosa	Malasia	Sea Sponge	alcoholic	+	-	-	-	-	-	-	-	Ibrahim et al., 2018
Pseudomonas fluorescens | H40, H1	Brazil	Sea Sponge	alcoholic	-	+	-	-	+	-	+	-	Nunes et al., 2021
Pseudomonas sp.	Italia	Endophytic	Free cell	+	+	-	-	-	-	-	-	Calonico et al., 2021
Pseudomonas sp. | AALPS.10. MNAAK.13	Canada	Ice desert (Artic)	alcoholic	+	-	-	-	-	-	-	+	Marcolefas et al., 2019
Pseudomonas sp. | RHLB12	India	Sea Sponge	alcoholic	+	-	-	-	-	-	-	-	Skariyachan et al., 2013
Saccharothrix sp. | 16Sb2-4	China	Hot desert	alcoholic	+	-	+	-	+	+	+	-	Liu et al., 2021
Salinispora sp. | 33'5	Mexico	Marine Sediment	culture filtrate	-	+	-	+	+	+	+	-	Contreras-Castro et al., 2020
Salinispora sp. | 9'4	Mexico	Marine Sediment	culture filtrate	-	+	-	+	+	+	+	-	Contreras-Castro et al., 2020
Salinispora sp. | FS-0034	Fiji	Marine Sponge	alcoholic	+	-	+	-	-	-	-	-	Singh et al., 2014
Serratia marcescens | JSSCPM1	India	Soil	alcoholic	+	-	-	-	-	-	-	-	Arivuselvam et al., 2023

TABLE 2 - ANTIBACTERIAL ACTIVITY OF ENVIRONMENTAL ISOLATES AGAINST ESKAPEE PATHOGENS, ACCORDING TO THE COUNTRY OF ORIGIN, SOURCE, AND EXTRACT (Continuation)

Specie | Strain	Country	Source	Extract	Antibacterial activity								Reference
				MRSA	MDS	VRE	MDRE	MDRP	MDRA	MDK	MDREc	
Streptomyces agglomeratus | 5-1-3	China	Mountain (High altitude)	alcoholic	-	+	-	-	-	-	-	-	Jiang et al., 2023
Streptomyces albidoflavus | CMRP4854	Brazil	Endophytic	alcoholic	-	-	-	-	-	+	-	-	Assad et al., 2021
Streptomyces albogriseolus | 6BTZ-4	China	Endophytic	water	-	+	-	-	+	-	+	-	Jiang et al., 2018
Streptomyces anulatus	Iran	Soil	alcoholic	+		-	-		-	-	-	Ghashghaei et al., 2018
Streptomyces bacillaris | RAM25C4	India	Marine Sediment	alcoholic	+	+	-	-	+	+	-	-	Wahaab et al., 2018
Streptomyces bingchenggensis ULS14	Nigeria	Soil	alcoholic	+	-	-	-	-	-	-	-	Flora et al., 2015
Streptomyces chryseus	Iran	Soil	alcoholic	+	-	-	-	-	-	-		Ghashghaei et al., 2018
Streptomyces coelicoflavus | EMM112	Morocco	Marine Sediment	Free cell	+	-	-	-	-	-	-	+	Ibrahimi et al., 2020
Streptomyces erythrogriseus | M10-77	Peru	Marine Sediment	alcoholic	+	+	+	-	-	-	-	-	Leon et al., 2011
Streptomyces fulvissimus | ULK2	Nigeria	Soil	alcoholic	+	-	-	-	-	-	-	-	Flora et al., 2015
Streptomyces gandocaensis | DHS287	Costa Rica	Marine Sediment	alcoholic	-	-	-	-	-	+	-	-	Park et al., 2016
Streptomyces griseus | TAAAP033	India	Industrial wastewater	alcoholic	-	-	-	+	+	-	-	-	Kumar et al., 2021
Streptomyces longispororuber | SBRK2	India	Sea Sponge	alcoholic	+	-	-	-	-	-	-	-	Mary et al., 2021
Streptomyces omiyaensis | SUK 25	Malasia	Endophytic	alcoholic	+	-	-	-	-	-	-	-	Alshaibani et al., 2016
Streptomyces parvulus | Av-R5	India	Endophytic	alcoholic	-	+	-	-	+	-	+	+	Chandrakar et al., 2019

TABLE 2 - ANTIBACTERIAL ACTIVITY OF ENVIRONMENTAL ISOLATES AGAINST ESKAPEE PATHOGENS, ACCORDING TO THE COUNTRY OF ORIGIN, SOURCE, AND EXTRACT (Continuation)

Specie | Strain	Country	Source	Extract	Antibacterial activity								Reference
				MRSA	MDS	VRE	MDRE	MDRP	MDRA	MDK	MDREc	
Streptomyces phaeoluteichromatogenes | 7BMP -1	China	Endophytic	mycelium	-	+	+	+	-	-	-	-	Jiang et al., 2018
Streptomyces rochei | PM49	India	Marine Sediment	alcoholic	-	+	-	-	-	-	-	-	Shanthi et al., 2015
Streptomyces smyrnaeus | UKAQ 23	Saudi Arabia	Marine Sediment	alcoholic	+	-	-	-	-		-	-	Qureshi et al., 2021
Streptomyces sp. | Al-Dhabi-90	Saudi Arabia	Marine Sediment	alcoholic	+	-	-	-	+	+	-	+	Al-Dhabi et al., 2019
Streptomyces sp. | CJ13	Ireland	Soil	alcoholic	+	-	-	-	-	-	-	-	Quinn et al., 2021
Streptomyces sp. | DSD011	Philippines	Marine Sediment	alcoholic	+	+	-	-	-	-	-	-	Sabido et al., 2020
Streptomyces sp. | MW562807	Peru	Saline Lagoon	alcoholic	-	-	-	-	-	+	-	-	Clavo et al., 2021
Streptomyces sp. | PS95	Thailand	Soil	Free cell	+	-	-	-	-	-	-	-	Chanthasena et al., 2016
Streptomyces sp. | RO-S4	Argelia	Marine Sediment	alcoholic	+	-	-	-	-	-	-	-	Ouchene et al., 2022
Streptomyces sp. | S3	Argelia	Marine Sediment	Free cell	+	-	-	-	-	-	-	-	Ouchene et al., 2022
Streptomyces sp. | SA 11	Saudi Arabia	Marine Sediment	alcoholic	+	-	-	-	-	-	-	-	Al-Ansari et al., 2020
Streptomyces sp. | SA32	Indonesia	Endophytic	alcoholic	+	+	+	+	-	-	+	+	Ryandini et al., 2021
Streptomyces sp | TAAAP012	India	Industrial wastewater	alcoholic	-	-	-	+	+	-	-	-	Kumar et al., 2021
Streptomyces sundarbansensis | 3BXP - 1	China	Endophytic	water extract	-	-	-	-	+	-	-	+	Jiang et al., 2018
Thermomonas hydrothermalis | H1	Jordan	Hot spring	alcoholic	-	+	+	-	-	-	-	-	Al-Daghistani et al., 2021

TABLE 2 - ANTIBACTERIAL ACTIVITY OF ENVIRONMENTAL ISOLATES AGAINST ESKAPEE PATHOGENS, ACCORDING TO THE COUNTRY OF ORIGIN, SOURCE, AND EXTRACT (conclusion)

Specie | Strain	Country	Source	Extract	Antibacterial activity								Reference
				MRSA	MDS	VRE	MDRE	MDRP	MDRA	MDK	MDREc	
Verrucosispora sp. | CMRP4860	Brazil	Endophytic	alcoholic	+	-	+	-	-	-	-	-	Assad et al., 2021
Virgibacillus salarius	Indonesia	Sea slug	alcoholic	+	-	-	-	-	-	-	-	Kristiana et al., 2020

SOURCE: The author (2024).

Legend: (+) the extract or cell-free was tested against bacteria with a known resistance profile; (-) negative result against resistant bacterial pathogens or bacterial extract has not been tested MRSA = Methicillin Resistance *Staphylococcus aureus*; MDRSA = Multidrug Resistance *Staphylococcus aureus*; VRE = Vancomycin Resistance *Enterococcus*; MDRE = Multidrug Resistance *Enterococcus*; MDRP = Multidrug Resistance *Pseudomonas*; MDRA = Multidrug Resistance *Acinetobacter baumannii*; MDRK = Multidrug Resistance *Klebsiella*; MDREc Multidrug Resistance *Escherichia coli.*

2.4.3 Antibacterial chemical compounds present in the different extracts and the analytical methods employed

The 64 bacterial extracts were tested for antimicrobial activity (Table 2), but only 33 of these extracts were characterized (Table 3). Analyzing the activity of individual substances revealed new and old compounds with activity against MDR bacteria. For example, echinomycin produced by *Streptomyces agglomeratus* inhibited MDRS (Jiang et al., 2023), rifamycin W produced by *Salinispora* spp. inhibited MRSA and VRE (Singh et al., 2014), actinomycin X2 and actinomycin D produced by *Streptomyces* inhibited MDRSA, MDRP, MDRK, and MDREc (Chandrakar et al., 2019; Qureshi et al., 2021) (Table 3).

Some strains were also associated with the production of new compounds. These compounds showed inhibitory activity against MDR pathogens. For example, fridamycin A and fridamycin D produced by *Streptomyces* can inhibit MRSA and MDRS (Sabido et al., 2020; Ouchene et al., 2022). Baoshanmycin, produced by *Amycolatopsis*, can inhibit MDRS strains of *S. aureus* (Qian et al., 2022); pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro, produced by *Bacillus tequilensis* MSI45, has activity against MRSA and VRE (Kiran et al., 2018). Engelhardt et al. (2010) described a new thiopeptide antibiotic produced by *Nocardiopsis* that can inhibit VRE.

Among the 25 bacterial extracts whose identified compounds exhibited antibacterial activity (Table 3), 12 extracts were subjected to HPLC-MS (high-performance liquid chromatography coupled with mass spectrometry) with or without methodological variations such as high-resolution (HR), electrospray ionization (ESI), and time-of-flight (TOF) mass spectrometry (Qian et al.; 2022; Chakraborty et al., 2021; Chakraborty et al., 2021; Back et al., 2023; Hamedi et al., 2015; Engelhardt et al., 2010; Kristiana et al., 2020; Liu et al., 2021; Singh et al., 2014; Park et al., 2016; Kumar et al., 2021; Mary et al., 2021; Chandrakar et al., 2019; Shanthi et al., 2015; Qureshi et al., 2021; Sabido et al., 2020). The remaining 10 of the 12 samples were analyzed using alternative spectroscopic techniques, including nuclear magnetic resonance (NMR), Fourier transform infrared (FT-IR), and gas chromatography coupled with mass spectrometry (GC-MS). The remaining bacterial extracts were characterized using the following methods: HPLC (Kumar et al., 2021); NMR (Chakraborty et al., 2021); GC-MS (Alshaibani et al., 2016; Al-Dhabi et al., 2019); FT-IR (Skariyachan et al., 2014);

HPLC and NMR (Jiang et al., 2023); NMR, FT-IR, and GC-MS (Kiran et al., 2018; Raja et al., 2023).

2.4.4 Intellectual properties of chemical compounds with antibacterial activity

In total, 19 patents were found for 9 of the 25 bacterial extracts with characterized compounds (Table 3); the patents originated from six countries (USA, China, South Korea, Brazil, Italy, and France). Moreover, among the 25 bacterial extracts used to characterize the compound(s) responsible for the observed antibacterial activity, 21 biocompounds are commercially available and are widely used in the pharmaceutical and biotechnology industries (Table 3). A total of 7 of these commercially available biocompounds can be used in different forms (tablets, sprays, creams and liquids), such as carbenicillin, cephalexin, cephalothin, tetracycline, chloramphenicol, actinomycin D and 8-O-methyltetrangomycin. In contrast, 14 compounds are used exclusively for research, namely: 3-methylpyridazine, n-hexadecanoic acid, fridamycin A and D, cislabdan sulfanil-type compounds, cyclo(L-Pro-L-Trp), actinomycin X0β, pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro, isoquinocycline B, stearidonic acid, prodigiosin, aldgamycin G, rifamycin W, and echinomycin (NCBI, 2024.

TABLE 3 - DESCRIPTION OF THE CHEMICAL COMPOUNDS DETECTED IN THE SECONDARY METABOLITES OF EXTRACTS OF ENVIRONMENTAL BACTERIA WITH ANTIBIOTIC ACTIVITY AGAINST ESKAPEE PATHOGENS AND THE ANALYTICAL METHOD USED (Continue)

Specie | Strain	Compound	Analytical Method	Patent code	Commercialized Producer	Reference
Amycolatopsis sp. | YNNP 00208	Baoshanmycin ($C_{17}H_{19}NO_7$)	$^1H/^{13}C$ NMR HRESIMS	-	-	Qian et al., 2022
Bacillus amyloliquefaciens | MTCC 12713	Hexahydro-41-hydroxy-macrobrevin-31-acetate	NMR FT-IR LC-MS GC-MS	-	-	Qian et al., 2022
Bacillus amyloliquefaciens | MTCC 12716	Amylomacin B (amicoumacin compound encompassing 4′-hydroxy-11′-methoxyethyl carboxylate functionality)	$^1H/^{13}C$ NMR GC-MS HRESIMS FT-IR	-	-	Chakraborty et al., 2021
Bacillus sp. | DJ4 **	Baptifoline, Dehydromorroniaglycone, Isoleucinopine, Sophoramine, Melazolide A, Rengyoside C, Iedoglucomide A, Paenilamicin A1*, Aeruginopeptin	LC-MS/MS Xevo G2-XS QTof; ESI	-	-	Priyanto et al., 2023
Bacillus sp. | DJ9 **	Baptifoline, Dehydromorroniaglycone, Isoleucinopine, Sophoramine, Aspersecosteroid A, Rengyoside C, Iedoglucomide A, Paenilamicin A1*, Aeruginopeptin	LC–MS/MS Xevo G2-XS QTof; ESI	-	-	Priyanto et al., 2023
Bacillus subtilis| TAAAP010	Carbenicillin	RP-HPLC	-	Yes	Kumar et al., 2021
	Cephalexin		-	Yes	
	Cephalothin		-	Yes	
	Tetracycline		-	Yes	
Bacillus tequilensis | MSI45	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro	FT-IR NMR GC-MS	-	Yes	Kiran et al., 2018
Bacillus velezensis | MTCC13048	Amido-type 12-membered macrocyclic polyketide, 8-(pent-2-enyl)-1-oxo-5a, 8a-dioxacyclododecanyl-3-oxy-ethyl-5b′-methyl-5′-(7′(9′(methoxyethyl)-dihydrofuranyl) propanmido) succinate ($C_{32}H_{47}NO_{12}$)	$^1H/^{13}C$ NMR	-	-	Chakraborty et al., 2021
Micromonospora marina | *KPMS1*	(2E) -3-(2H-1,3-benzodioxol-5-yl) -N- phenyl prop-2-enamide ($C_{16}H_{13}NO_3$)	FT-IR $^1H/^{13}C$ NMR	-	-	Raja et al., 2023

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Specie | Strain	Compound	Analytical Method	Patent code	Commercialized Producer	Reference
Micromonospora robiginosa | 28ISP2-46	Isoquinocycline B	LC–MS	-	Yes	Back et al., 2023
Nocardia sp. | UTMC 751	Unknown compounds (molecular weights of 274.2, 390.3, 415.3, 598.4 and 772.5 D)	HPLC-MS	-	-	Hamedi et al., 2015
Nocardiopsis sp. | TFS65-07	Thiopeptide antibiotic TP-1161	HPLC-DAD-TOF $^{1}H/^{13}C$ NMR HRMS	-	-	Engelhardt et al., 2010
Pseudoalteromonas rubra	Stearidonic acid ($C_{18}H_{28}O_{2}$)		US2020352980A (USA)	Yes	
	Prodigiosin, ($C_{20}H_{25}N_{3}O$)	HPLC–MS	US2017058314A1(USA) US2021123086A1(USA) CN105603737A(China) BR1020210232625 (Brazil)	Yes	Kristiana et al., 2020
Pseudomonas sp. | RHLB12	Chromophoric substance	FT-IR	-	-	Skariyachan et al., 2014
Saccharothrix sp. | 16Sb2-4	Aldgamycin H	UPLC-QToF-MS/MS; $^{1}H/^{13}C$ NMR	-	-	Liu et al., 2021
	Aldgamycin K		-	-	
	Aldgamycin G ($C_{37}H_{56}O_{15}$)		EP2202293A2 (France) US2010120896A1(USA) US2022021922(USA) US2019067914(USA)	Yes	
	Swalpamycin B		-	-	
Salinispora sp. | FS-0034	Rifamycin W ($C_{35}H_{45}NO_{11}$)	HRESIMS ^{1}H NMR	IB2010051183 (Italy) US2022168384A1(USA) US2020263224A1(USA) KR102342719B1 (South Korea)	Yes	Singh et al., 2014
Streptomyces agglomeratus | 5-1-3	Echinomycin ($C_{51}H_{64}N_{12}O_{12}S_{2}$)	HPLC; NMR	WO2024002385A1 (China)	Yes	Jiang et al., 2023
Streptomyces bacillaris | RAM25C4 **	2,6-di-tert-butylphenol; 1H, 5H, Pyrrolo (1′ 2′:3, 4) imidazole; 1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl)	HPTLC GC–MS	-	-	Wahaab et al., 2018

TABLE 3 - DESCRIPTION OF THE CHEMICAL COMPOUNDS DETECTED IN THE SECONDARY METABOLITES OF EXTRACTS OF ENVIRONMENTAL BACTERIA WITH ANTIBIOTIC ACTIVITY AGAINST ESKAPEE PATHOGENS AND THE ANALYTICAL METHOD USED (Continuation)

Specie | Strain	Compound	Analytical Method	Patent code	Commercialized Producer	Reference
Streptomyces fulvissimus | ULK2	Amythiamicins*	GS	-	-	Flora et al., 2015
Streptomyces bingchenggensis ULS14	Amythiamicins*	GS	-	-	Flora et al., 2015
Streptomyces gandocaensis | DHS287	Cahuitamycins A ($C_{27}H_{37}N_7O_{11}$) and C ($C_{28}H_{39}N_7O_{11}$)	NMR HPLC-MS	US10239918B2 (USA)	-	Park et al., 2016
Streptomyces griseus | TAAAP033	Carbenicillin	RP-HPLC	-	Yes	Kumar et al., 2021
	Cephalexin		-	Yes	
	Cephalothin		-	Yes	
	Tetracycline ($C_{22}H_{24}N_2O_8$)		CN20120010019 (China)	Yes	
Streptomyces longispororuber | SBRK2	8-O-metyltetrangomycin ($C_{20}H_{16}O_5$)	HR-LC-MS	EP3303340 (Netherlands)	Yes	Mary et al., 2021
Streptomyces omiyaensis | SUK 25	Cyclo(L-Pro-L-Trp)	GC–MS	-	Yes	Alshaibani et al., 2016
	Chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$)		KR20230001082A (South Korea)	Yes	
Streptomyces parvulus | Av-R5	Actinomycin D ($C_{62}H_{86}N_{12}O_{16}$)	$^1H/^{13}C$ NMR FT-IR	CN104450580A (China) CN111778178B (China)	Yes	Chandrakar et al., 2019
	Actinomycin X0β	HESI-MS	-	Yes	
Streptomyces rochei | PM49	Sulfanyl cyslabdan-like compound ($C_{25}H_{41}NO_5S$)	FT-IR HESI-MS $^1H/^{13}C$ NMR	-	Yes	Shanthi et al., 2015
Streptomyces smyrnaeus | UKAQ 23	Actinomycin X2	LC-MS NMR	-	-	Qureshi et al., 2021
	Actinomycin D ($C_{62}H_{86}N_{12}O_{16}$)		CN104450580A (China) CN111778178B (China)	Yes	
Streptomyces sp. | Al-Dhabi-90	3-methylpyridazine	GC–MS	-	Yes	Al-Dhabi et al., 2019
	n-hexadecanoic acid		-	Yes	
Streptomyces sp. | DSD011	Fridamycin A	LCMS-TOF MS/MS	-	Yes	Sabido et al., 2020
	Fridamycin D		-	Yes	

TABLE 3 - DESCRIPTION OF THE CHEMICAL COMPOUNDS DETECTED IN THE SECONDARY METABOLITES OF EXTRACTS OF ENVIRONMENTAL BACTERIA WITH ANTIBIOTIC ACTIVITY AGAINST ESKAPEE PATHOGENS AND THE ANALYTICAL METHOD USED (Conclusion)

Specie | Strain	Compound	Analytical Method	Patent code	Commercialized Producer	Reference
Streptomyces sp. | MW562807 **	Cholic Acid; Lobophorin A, B, E, and K; in addition to a Sixth Compound	UHPLC-MS	-	-	Clavo et al., 2021
Streptomyces sp. | RO-S4	Aquayamycin *	UHPLC-HRMS/MS	-	-	Ouchene et al., 2022
Streptomyces sp. | TAAAP012	Carbenicillin	RP-HPLC	-	Yes	Kumar et al., 2021
	Cephalexin		-	Yes	
	Cephalothin		-	Yes	
	Tetracycline ($C_{22}H_{24}N_2O_8$)		CN20120010019 (China)	Yes	
Thermomonas hydrothermalis | H1 **	l-ornithine, 5-aminocarbonyl-α-amino-ureidovaleric acid (citrulline), tetramethyl-2-hexadecene, 2-hexyl-1,4-trimethylcyclobutane, glycyl-L-proline, pyrrole 1,2 pyrazine-1,4 dione, 2-hydroxy-trimethyl cyclohexanone, pyrolopyrazine-1,2-pyrazine-1,4-dion, 5-nitroso-2,4,6-triamino pyrimidine, pyrimidinone, octadecanoic acid, actinomycin D (cyclic peptide), 2-ethoxy-4,7-dimethylpyrido pyrimidin, octahydro-1H-pyridopyrimidin, ergotaman, octa-decanoic acid, tropyl propanal tosyl hydrazone, and dihydroxy-1,5-naphthyridine	GC–MS	-	-	Al-Daghistani et al., 2021

SOURCE: The author (2024).

Legend: (-) The search criteria yielded no patents and no commercially available products containing the specified compounds. (*) Antibacterial compound suggested by the author; (**) Compounds characterized but not proven to have antibacterial activity. GC - Gas chromatography; HRESIMS -High-resolution electrospray ionization mass spectrometry; NMR - nuclear magnetic resonance; FT-IR - Fourier transform infrared spectroscopy; GC–MS - gas chromatography-mass spectrometry; RP-HPLC - Reverse Phase High Performance Liquid Chromatography; UHPLC-HRMS/MS - ultra-high-performance liquid chromatography with high-resolution mass spectrometry; TOF MS/MS Quadrupole time-of-flight mass spectrometry; DAD- Diode Array Detector.

2.5 DISCUSSION

In this review, the data indicated that the marine environment represents the most extensively investigated niche where bacteria with MDR antimicrobial potential have been isolated (Asagabaldan et al., 2017; Al-Dhabi et al., 2019; Contreras-Castro et al., 2020). Microorganisms that can survive extremely hostile conditions may produce secondary metabolites to suppress their ecological competitors (Schultz et al., 2023). This phenomenon was found in marine sponges, sea slugs, and algae with their microbial symbiosis, which represents a unique ecological phenomenon and acts as a reservoir of new bioactive molecules (Singh et al., 2014; Skariyachan et al., 2014; Asagabaldan et al., 2017; Ibrahim et al., 2018; Kiran et al., 2018; Back et al. 2021; Chakraborty et al., 2021; Mary et al., 2021; Chakraborty et al., 2022).

Over the past two decades, the discovery of previously characterized bioactive compounds and the redundancy of strains have significantly reduced interest in soil-dwelling bacteria as a source of new bioactive compounds. Consequently, microorganisms that inhabit other niches, such as marine environments, have gained popularity due to their chemical diversity (De La Hoz-Romo et al., 2022). Several environmental fluctuations affect marine microorganisms, including temperature, pressure, light, and salinity, all of which facilitate the biosynthesis of distinctive metabolites (Stincone and Brandelli, 2020).

In other extreme environments, such as those with water deficiency (hot and ice deserts), high osmotic pressure (saline lagoons), or high temperatures (hot springs), various microorganisms can also produce unknown secondary metabolites (Hamedi et al., 2015; Efimenko et al., 2018; Marcolefas et al., 2019; Saadatpour and Mohammadipanah, 2020; Al-Daghistani et al., 2021; Clavo et al., 2021; Liu et al., 2021). For example, *Streptomyces* is a gram-positive bacterial genus that can produce various compounds with antimicrobial activity, including streptomycin, chloramphenicol, and tetracycline (Schlimpert et al., 2023; Meenakshi et al., 2024). In this context, the reviewed studies showed a high diversity of *Streptomyces*, presented from all clades of this genus that were not closely related, according to the phylogenetic analysis (Labeda et al., 2017), which revealed that all species from the genus can produce antimicrobial substances. Other highly expressed species associated with these extreme environments are those belonging to the genera *Bacillus*, *Chromohalobacter*, *Nocardia*, *Nocardiopsis*, *Pseudomonas, Saccharothrix,* and

Salinispora, which also produce various antimicrobial substances, including polypeptides and lipopeptides (Engelhardt et al., 2010; Hamedi et al., 2015; Asagabaldan et al., 2017; Efimenko et al., 2018; Marcolefas et al., 2019; Liu et al., 2021).

This review highlighted that several compounds previously identified with antimicrobial activity can be further emerged as new classes for treating ESKAPEE pathogen infections (Sabido et al., 2020; Qian et al., 2022). For example, the thiopeptide antibiotic produced by *Nocardiopsis* sp. is very similar to the substance A10255, which is used as an animal growth promoter and has high activity against VRE (Engelhardt et al., 2010). Prodigiosin, present in the extracts of *P. rubra* and *V. salaries* (Kristiana et al., 2020), functions as an antimicrobial agent (bacteria, fungi, and viruses), a biological pigment, a bacterial metabolite, an apoptosis inducer, and an antineoplastic agent, although it is still in the experimental phase (Choi et al., 2021). Paenilamicin A1, present in the extracts of *Bacillus* sp. DJ4 and DJ9 (Priyanto et al., 2023), was described as a secondary metabolite antibiotic produced by the honeybee pathogenic bacterium *Paenibacillus larvae* and showed antibacterial, antifungal, and cytotoxic activity (Garcia-Gonzalez et al., 2014). Fridamycin A, isolated from *Streptomyces* sp. (Sabido et al., 2020; Ouchene et al., 2022), showed activity against MRSA and VRE. It is also a new candidate for treating type 2 diabetes by stimulating glucose uptake without inducing adipogenesis through the activation of the AMPK signaling pathway (Yoon et al., 2019).

Another previously uncharacterized substance has shown an abundance of new compounds with antimicrobial potential, such as the compound pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro produced by *B. tequilensis*, which exhibits activity against MRSA and VRE (Kiran et al., 2018) and has low cytotoxicity (Kannabiran et al., 2014). Cahuitamycins A-C produced by *Streptomyces gandocaensis* DHS287 were recently described and shown to have antibiofilm activity against *Acinetobacter* (Park et al., 2016), and a compound called cyclo (L-Trp L-Pro), produced by *Streptomyces omiyaensis* SUK 25, possesses activity against MRSA and *Micrococcus luteus* (Mehdi et al., 2009; Alshaibani et al., 2016). This review also described the production of previously known substances, with only some chemical alterations, which can lead to new activities against previously resistant bacteria. For example, rifamycin W, which is an analog of rifamycin SV and has high activity against MDRB (Adams et al., 2021), is produced by *Salinispora* sp. FS-0034 (Singh et al., 2014). Actinomycin D and

actinomycin X are produced by *Streptomyces* sp. (Chandrakar et al., 2019; Qureshi et al., 2021) and are well-known antibiotics that exhibit high antibacterial and antitumor activity (Finocchiaro, 2020).

The analytical techniques used to characterize bioactive compounds in bacterial extracts were discussed. Among the 25 extracts studied, 12 were analyzed mainly via high-performance liquid chromatography-mass spectrometry (HPLC-MS), with two extracts being analyzed exclusively using this method (Back et al., 2023; Kristiana et al., 2020) (Table 3). LC-MS is a popular technique in metabolomic studies because of its versatility and simplicity (Canuto et al., 2018; Alseekh et al., 2021). Moreover, LC-MS, other techniques, such as NMR, GC-MS, and FT-IR, were used to confirm the structural characteristics of the bioactive compounds. NMR is a reliable technique that requires minimal sample handling but has lower sensitivity and selectivity. GC-MS is widely used in metabolomic studies, but metabolites generally need to be volatilized for analysis at lower temperatures (Schrimpe-Rutledge et al., 2016; Alseekh et al., 2021). Finally, FT-IR was used occasionally in some studies (Neto et al., 2022). Overall, the combination of these analytical techniques provides a comprehensive understanding of the bioactive compounds found in bacterial extracts. Moreover, the cytotoxicity of the compounds was evaluated in only 5 reports (Al-Ansari et al., 2020; Kristiana et al., 2020; Saadatpour and Mohammadipanah, 2020; Al-Daghistani et al., 2021; Back et al., 2021).

Considering the antibacterial activity observed in the characterized compounds, this study also identified the primary patent holders and their geographical distribution by analyzing patent publications without considering the commercial status of patents. As determined by the patent survey of bioactive compounds, the United States holds the greatest number of patents (9/20) for five antibacterial compounds (stearidonic acid, prodigiosin, aldgamycin G, rifamycin W, and cahuitamycins A–C (Table 3), followed by China and South Korea. However, some authors have indicated that China is the predominant patent holder for commercial and non-commercial antibacterial agents. Besides China, the United States, Japan, South Korea, India, the United Kingdom, and Italy have prominent commercial patents (Jimenez et al, 2022; Krittika et al, 2024).

In this context, the data gathered from the articles under review indicate that bioprospecting bacteria of environmental origin may represent a promising strategy to address the challenge of MDR bacteria. The potential discovery of novel bioactive

compounds could offer valuable tools to enhance the efficacy of existing treatments for bacterial infections, which have contributed to significant mortality rates. Furthermore, to enhance the development of new antibacterial agents and expedite the process, it is essential to refine and advance the experimental protocols employed in these studies. In particular, the characterization of compounds present in bacterial extracts requires more comprehensive and rigorous methodology.

CONFLICT OF INTERESTS

The authors declare no competing interest.

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3 CHAPTER II

EVALUATION OF MULTIDRUG RESISTANT PROFILE OF CLINICAL BACTERIA AT THE CLINICAL HOSPITAL COMPLEX FROM THE FEDERAL UNIVERSITY OF PARANA IN CURITIBA, BRAZIL

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3.1 ABSTRACT

The prevalence of multidrug resistant (MDR) bacteria represents a significant challenge to global health, leading to a lack of treatment options and elevated morbidity and mortality rates. The emergence and spread of these resistant strains necessitate continuous surveillance and in-depth investigations to ascertain their prevalence and the mechanisms underlying their resistance. The present study is concerned with the evaluation of the multidrug resistant (MDR) profile of 65 clinical bacteria isolated from the Hospital Complex of the Federal University of Paraná (CHC/UFPR) in Curitiba, Brazil. The objective is to characterize the resistance profiles of bacterial strains that have been identified by the World Health Organization (WHO) as priority resistant bacteria in the search for new treatments. Furthermore, the objective is to furnish data on microbiological collection in bioprospecting studies. The isolates were subjected to MALDI-TOF identification, followed by antibiotic susceptibility testing using disk diffusion screening and molecular identification of resistance genes using PCR. The findings elucidate the resistance profiles of pathogens that have been circulating in the hospital from 2011 to 2021, thereby contributing to global efforts to monitor and mitigate resistance. Moreover, the microorganisms deposited in the Center for Microbiological Collections of the Paraná Taxonline Network (CMRP/Taxonline) can serve as a valuable resource for future studies on drug resistance. This study highlights the necessity of implementing personalized strategies to enhance patient outcomes and antibiotic stewardship programs in the context of combating multidrug resistant (MDR) bacteria.

Keywords: ESKAPEE; antimicrobial susceptibility; resistance gene; bacteria.

3.2 INTRODUCTION

Multidrug resistant (MDR) bacteria represent a significant challenge in clinical settings worldwide, posing a severe threat to public health due to limited treatment options and increased morbidity and mortality (Talaat et al., 2022). The emergence and spread of these resistant strains necessitate ongoing surveillance and comprehensive studies to understand their prevalence and resistance mechanisms. In this context, teaching hospitals serve as critical centers for monitoring and evaluating MDR pathogens, given their role in treating a diverse patient population and their involvement in cutting-edge research and clinical practices (Zagui et al., 2021).

The efficacy of existing antibiotics is on the decline, primarily due to the proliferation of resistant strains causing infections (Sanchez-Urtaza et al., 2023). Resistance mechanisms, such as reduced drug entry, are increasingly observed in bacteria like Enterobacterales and *Pseudomonas aeruginosa*, impacting antibiotics like penicillins, cephalosporins, aminoglycosides, and tetracyclines (Panditt al., 2020). Notably, the prevalence of beta-lactamase resistance has risen significantly across various bacterial species. Also, the emergence of multidrug resistant (MDR) phenotypes further compounds the therapeutic challenge, severely constraining available treatment options for antibiotic-resistant organisms (Nawaz et al., 2009).

The antibiotic pipeline criterion was given a higher weight than in WHO (2017) (Asokan et al., 2019). This shows experts recognize the importance of developing new antibiotics. This may show experts recognize the limited progress in developing new drugs to combat priority pathogens. In 2023, a WHO report said that the pipeline was not moving forward. This shows that its need to invest in research and development to find new and effective treatments (Avershina et al., 2021).

Furthermore, in 2024 the World Health Organization (WHO) published a list of global research priorities to treat multidrug resistant (MDR) and extensively drug-resistant (XDR) bacteria. The list of priority pathogens includes Enterobacterales carbapenem-resistant, Enterobacterales third-generation cephalosporin-resistant, *Acinetobacter baumannii* carbapenem-resistant and *Mycobacterium tuberculosis*, rifampicin-resistant. These bacteria can cause serious and often fatal infections, including bloodstream infections and pneumonia. In addition, the list of high groups: *Salmonella Typhi* fluoroquinolone-resistant; *Shigella* spp. fluoroquinolone-resistant; *Enterococcus faecium* vancomycin-resistant; *Pseudomonas aeruginosa* carbapenem-

resistant; Non-typhoidal *Salmonella* fluoroquinolone-resistant; *Neisseria gonorrhoeae* third-generation cephalosporin, and/or fluoroquinolone-resistant, and Methicillin-resistant *Staphylococcus aureus* (WHO, 2024).

Other leading organizations report that, for example, the 2022 Global Antimicrobial Resistance and Use Surveillance System (GLASS) report highlights alarming resistance rates among prevalent bacterial pathogens. Median reported rates in 76 countries of 42% for third-generation cephalosporin-resistant *E. coli* and 35% for methicillin-resistant *Staphylococcus aureus* are a major concern. For urinary tract infections caused by *E. coli*, 1 in 5 cases exhibited reduced susceptibility to standard antibiotics like ampicillin, co-trimoxazole, and fluoroquinolones in 2020. This is making it harder to effectively treat common infections (WHO, 2023).

Therefore, the bacterial species analyzed in this study are part of a group known as ESKAPEE. "ESKAPEE" comes from the first letters of several bacterial names: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli* and *Enterobacter* species, also known as super bacteria, which are an important group of highly drug-resistant bacteria commonly seen in hospitals (Xuan et al., 2023).

The present study focused on evaluating the multidrug-resistant profile of 65 clinical isolates. Through phenotypic and molecular analyses providing information on the resistance profiles of pathogens circulating in the hospital from 2011 to 2021 and contributing to the global effort to monitor the emergence of new resistance and mitigate the impact of multidrug-resistant bacteria. Furthermore, the microorganisms analyzed in the present work were deposited at the Microbiological Collections Center of the Paraná Taxonline Network (CMRP/Taxonline) and can be used for future studies on drug resistance. Therefore, our findings support the development of targeted strategies to improve patient outcomes and antibiotic stewardship programs.

3.3 MATERIALS AND METHODS

3.3.1 Biological Samples

Sixty-five isolates of bacteria were obtained retrospectively from hospitalized patients. The isolates consist of both gram-positive and gram-negative bacteria derived from urine, blood, bone, sputum, tracheal aspirate, rectal and secretion. The strains

were obtained from the Clinical Hospital Complex of the Federal University of Paraná (CHC/UFPR) from 2011 to 2021. The strains are then deposited in the database. Microbiological Collections Centre of the Taxonline Network of Paraná (CMRP/Taxonline-https://www.cmrp-taxonline.com/catalogue). The isolates were preserved in a Trypticase Soy Broth (TSB) medium with 20% glycerol at − 80 °C.

3.3.2 Bacterial Identification using MALDI-TOF-MS

The bacterial isolates obtained were identified by the MALDI-TOF-MS technique (Matrix Assisted Laser Desorption Ionization Time of Flight Mass) using the Microflex MALDI-TOF-MS Biotyper system (Bruker Daltonics Inc., Germany) according to the manufacturer's recommendations. All the strains identified were confirmed with high reliability score values at the species level (score > 2.00) (Tsuchida and Nakayama, 2022).

3.3.3 Disk-Diffusion Screening

Antimicrobial susceptibility test was performed and interpreted using the European Committee of Antimicrobial Susceptibility Test guidelines (EUCAST, 2024). Disk diffusion was used as a screening test for the following antibiotics: ciprofloxacin, gentamicin, amikacin, cefepime, ceftazidime, imipenem and meropenem for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains. Ciprofloxacin, gentamicin, amikacin, cefepime, ceftriaxone, ertapenem and meropenem for Enterobacterales. Oxacillin, ciprofloxacin, gentamicin, clindamycin, erythromycin and sulfatrimethoprim for *Staphylococcus aureus*. ampicillin, ciprofloxacin, high-concentration gentamicin, high-concentration streptomycin, and vancomycin for *Enterococcus* sp.

3.3.4 DNA Extraction and Identification of Resistance Genes

The confirmation of the presence of resistance genes was performed by DNA extraction from the clinical isolates using the protocol previously described (Moreira et al., 2010). Then, a polymerase chain reaction was used to amplify 7 target genes associated with antibiotic resistance. Briefly, the reactions were carried out in a total

volume of 25 µL that consists of 1µL of DNA suspension (20-30ng) and 24 µL of PCR mixture: 1.5uL (50mM) MgCl2, 0.4uL (25mM) of dNTP (Invitrogen Inc., Carlsbad, CA), 2uL (50pmol) of each respective primer, and 0.5uL Platinum 5U Taq DNA polymerase (Invitrogen Inc., Carlsbad, CA) (Supplementary Material – S.M. 1).

The DNA amplification was performed in a GeneAmp PCR system 9700 Thermal Cycler (Applied Biosystems, Foster City, CA). A single PCR reaction condition was employed for each target gene: The *mecA* gene: 5 minutes at 94°C, followed by 30 cycles of 45 seconds at 94°C, 40 seconds at 55°C, and 45 seconds at 72°C, with a final extension of 10 minutes (Zhang et al., 2005); *vanA* gene: 5 minutes at 94ºC for the first cycle, followed by 30 cycles of 25 seconds at 94ºC, 40 seconds at 52ºC and 50 seconds at 72ºC, with a final extension of 6 minutes (Woodford and Stigter,1998); bla_{CTX-M} gene: 5 minutes at 94°C, followed by 35 cycles of 20 seconds at 94°C, 45 seconds at 58°C, and 45 seconds at 72°C, with a final extension of 6 minutes (Monteiro et al., 2012); bla_{OXA-23} gene: 5 minutes at 94°C, followed by 30 cycles of 25 seconds at 94°C, 40 seconds at 52°C, and 50 seconds at 72°C, with a final extension of 6 minutes (Woodford et al., 2006); $bla_{SPM,}$ bla_{NDM} and bla_{KPC} genes: 4 minutes at 94°C, followed by 35 cycles of 25 seconds at 94°C, 45 seconds at 58°C, and 45 seconds at 72°C, with a final extension of 6 minutes (Woodford et al., 2006).

3.4 RESULTS

3.4.1 Identification of Resistant Bacteria by MALDI-TOF-MS

According to identification through MALDI-TOF-MS , of the 65 strains, 10 bacterial species were identified, namely, *Acinetobacter baumannii* (N=9), *Citrobacter freundii* (N=2), *Klebsiella aerogenes* (N=1), *Enterobacter cloacae* (N=7), *Enterococcus faecium* (N=11), *Escherichia coli* (N=6), *Klebsiella oxytoca* (N=1), *Klebsiella pneumoniae* (N=9), *Proteus mirabilis* (N=1), *Pseudomonas aeruginosa* (N=6) and *Staphylococcus aureus* (N=12) (SM 2).

3.4.2 Disk-Diffusion Screening and Identification of Resistance Genes by *Polymerase Chain Reaction* (PCR)

Using disk-diffusion (DD) methodologies and the identification of resistance genes through PCR (S.M. 3), the results presented for the Enterobacterales strains (*Citrobacter freundii, Klebsiella aerogenes, Enterobacter cloacae, E. coli, Klebsiella oxytoca, K. pneumoniae e Proteus mirabilis)* were characterized as productive as ESBL producers (Extended Spectrum Beta-Lactamases) due to their resistance to the antibiotics analyzed by DD. Among of them *bla*$_{\text{CTX-M}}$ gene was detected in two *C. freundii* (CMRP6054 and CMRP6520), three *E. cloacae* (CMRP6522, CMRP6523, CMRP6524), three *E. coli* (CMRP6537, CMRP6539, CMRP6540), one *K. oxytoca* (CMRP6542), eight *K. pneumoniae* (CMRP6052, CMRP6053, CMRP6543, CMRP6545, CMRP6546, CMRP6547, CMRP6548, CMRP6549) and one *P. mirabilis* (CRMP6550). No *bla*$_{\text{CTX-M}}$ gene was detected in the other strains with an ESBL phenotype.

The carbapenemases genes were detected in *K. aerogenes* CMRP6521 (*bla*$_{\text{NDM}}$), *E. cloacae* CMRP6049 and CMRP6051 (*bla*$_{\text{KPC}}$) and *E. cloacae* CMRP6524 and CMRP6525 (*bla*$_{\text{NDM}}$) among the Gram-negative bacteria belonging to the order Enterobacterales that showed resistance to carbapenems. On the other hand, all the Gram-negative bacterial strains of *Acinetobacter baumannii* (N=9) and *Pseudomonas aeruginosa* (N=5) tested were resistant to carbapenems. The *A. baumannii* CMRP6056 and CMRP6517 strains amplified the (*bla*$_{\text{NDM}}$) gene. The *A. baumannii* strains CMRP6512, CMRP6513, CMRP6514, CMRP6515, CMRP6516, CMRP6518 and CMRP6519, CMRP6516, CMRP6518 and CMRP6519 amplified the *bla*$_{\text{OXA23}}$ gene. *P. aeruginosa* strains CMRP6057 (*bla*$_{\text{KPC}}$), *P. aeruginosa* CMRP6059 (*bla*$_{\text{SPM}}$), *P. aeruginosa* CMRP6553 (*bla*$_{\text{NDM}}$) and CMRP6554 (*bla*$_{\text{NDM}}$) were positive for at least one of the genes investigated (Appendix 1). Only one strain of *P. aeruginosa* did not amplify the carbapenemases genes through the specific PCR.

The Gram-positive strains *Staphylococcus aureus* (N=12) and *Enterococcus faecium* (N=11) were classified as resistant to methicillin and vancomycin, respectively. In addition, all *S. aureus* strains were positive for the *mec*A genes and all *E. faecium* strains amplified the *van*A gene.

3.5 DISCUSSION

In Brazil, the National Health Surveillance Agency (ANVISA), the Pan American Health Organization (OPAS), and the Ministry of Health have made efforts

to detect and control AMR since 2005, creating the AMR Network (Torumkuney et al., 2022). In 2018, Brazil began participating in the World Health Organization's Global Antimicrobial Resistance Surveillance System (GLASS) and published its National Action Plan on AMR, which established the need to create a national AMR surveillance program (BR-GLASS) (Keffir et al., 2023). In 2020, the BR-GLASS database contained more than 30,000 isolates.

Bacterial resistance to antibiotics is a pressing global health problem resulting from the natural evolution of bacteria and the overuse and misuse of antibiotics in various settings (Baran et al., 2023). This phenomenon has significant public health implications, as it undermines the effectiveness of standard treatments, increases healthcare costs, and prolongs recovery times (Correa et al., 2020).

The present study characterized the resistance profile of 65 clinical isolates of a variety of Gram-negative bacterial species belonging to the Enterobacterales (*C. freundii, K. aerogenes, E. cloacae, E. coli, K. oxytoca, K. pneumoniae,* and *P. mirabilis*), as well as strains of *A. baumannii, P. aeruginosa*, and Gram-positive strains of *E. faecium* and *S. aureus* (SM 2 and 3).

The strains showed a wide range of antibiotic susceptibility profiles and resistance genes, which were categorized as: carbapenem-resistant Enterobacterales, third-generation cephalosporin-resistant Enterobacterales, carbapenem-resistant *P. aeruginosa* and *A. baumannii*, methicillin-resistant *S. aureus*, and vancomycin-resistant *E. faecium*.

Considering the findings regarding the presence of carbapenem-resistant genes in this study, it can be posited that the 65 strains of multidrug resistant pathogenic bacteria align with the epidemiological statistics observed in Brazil. Among Enterobacterales, the most concerning resistance genes are *bla*$_{KPC}$ and *bla*$_{NDM}$, as evidenced by the publication by Keffir et al. (2023). Among the 27 Enterobacterales strains evaluated in this study, the *bla*$_{NDM}$ gene was predominant, occurring in four strains, while the *bla*$_{KPC}$ gene was present in two strains. Since its initial description in 2006 (Monteiro et al., 2009), the *bla*$_{KPC}$ gene has emerged as a significant concern among Enterobacterales in Brazil. Since 2015, data has indicated a decline in the prevalence of *bla*$_{KPC}$ among Enterobacterales, as well as among *K. pneumoniae*, *Citrobacter* spp., and *E. coli*. (WInk et al., 2021). The detection of *bla*$_{NDM}$ may indicate a potential replacement of carbapenemases in Brazil (Keffir et al., 2023; Arend et al., 2023.

Furthermore, 18 of the 27 Enterobacterales strains were extended-spectrum β-lactamases (ESBLs) producing, as they were positive for the presence of the *bla*$_{\text{CTX-M}}$ gene. ESBLs can hydrolyze all penicillins, cephalosporins, including those of the third and fourth generations, and monobactams. Over 500 distinct ESBLs have been identified, the majority of which are derived from the CTX-M, TEM, and SHV enzymes in Enterobacterales species (Bush; Jacoby, 2009). A recent resistome study of *E. coli* in Brazil revealed that the *bla*$_{\text{CTX-M}}$ gene is among the three most prevalent (Rodrigues et al., 2022).

About the carbapenemase genes identified in nine *A.baumannii* strains, the *bla*$_{\text{OXA23}}$ gene was detected in seven strains, followed by the *bla*$_{\text{NDM}}$ gene in two strains. The prevalence of the *bla*$_{\text{OXA23}}$ gene remains higher in Brazil (De Carvalho Hessel Dias et al., 2022). While less prevalent than *bla*$_{\text{OXA23}}$, the detection of b *bla*$_{\text{NDM}}$ in *Acinetobacter* spp. represents another noteworthy finding (Brasiliense et al., 2019).

The primary mechanisms of resistance of *P. aeruginosa* to carbapenems are overexpression of the efflux pump and overproduction of β-lactamase AmpC, which is associated with inactivation of the outer membrane protein OprD. However, the production of carbapenemases has become increasingly prevalent in this species (Tenover; Gill; Nicolau, 2022). While numerous carbapenemase genes have been identified globally in *P. aeruginosa*, the *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$ genes are the most prevalent (Tenover; Gill; Nicolau, 2022). Until recently, the *bla*$_{\text{SPM}}$ gene was the most prevalent in Brazilian isolates of *P. aeruginosa*. However, over the past decade, there has been a notable decline in the prevalence of *bla*$_{\text{SPM}}$ and an increase in the isolation of other carbapenemases, particularly *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, and *bla*$_{\text{VIM}}$, in select hospitals in Brazil (Kalluf et al., 2017)

Furthermore, the genes *van*A for *Enterococcus faecium* and *mec*A for *Staphylococcus aureus* were identified in 100% of the 11 and 12 strains, respectively, that were subjected to evaluation. The most clinically significant resistance phenotype among *Enterococcus* spp. is resistance to glycopeptides, particularly to vancomycin (also known as Vancomycin-Resistant *Enterococci*, or VRE). This resistance is associated with alterations in the biosynthesis of peptidoglycan precursors, which are mediated by the van genes, particularly *van*A and *van*B (Anvisa, 2020). The species most frequently implicated are *E. faecium* and *E. faecalis*. In the case of *Staphylococcus* spp., the most significant mechanism of resistance to β-lactams is associated with resistance to oxacillin/methicillin, which is mediated by the *mec*A gene.

This results in resistance to all betactams, except for the recently developed cephalosporins, ceftobiprole and ceftaroline (Anvisa, 2020).

Although the primary objective of this study was not to examine the epidemiology of the data, a preliminary overview of the 65 strains characterized suggests a representative sample of the current reality of public reference hospitals in Brazil about AMR. However, the primary objective was to provide crucial data for research in the pursuit of novel bioproducts or innovative therapeutic strategies against infections caused by drug-resistant microorganisms. The data will be deposited in microbiological collections that can be utilized as tools in strategies to contain bacterial resistance to antibiotics.

CONFLICT OF INTERESTS

The authors declare no competing interest.

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SUPPLEMENTARY MATERIAL 1 - CHARACTERISTICS OF THE PRIMERS USED IN THIS STUDY

Species	Primer	Sequence (5' to 3')	Size (pb)	Reference
Staphylococcus aureus	*mec*A - F	GTG AAG ATA TAC CAA GTG ATT	147	(Zhang et al., 2005)
	*mec*A - R	ATG CGC TAT AGA TTG AAA GGA T		
Enterococcus spp.	*van*A - F	ATGGCAAGTCAGGTGAAGATGG	200	(Woodfor,1998).
	*van*A - R	TCCACCTCGCCAACAACTAACG		
Enterobacterales	bla_{KPC} - F	TCGCTAAACTCGAACAGG	785	(Monteiro et al., 2012)
	bla_{KPC} - R	TTACTGCCCGTTGACGCCCAATCC		
Enterobacterales	bla_{NDM} - F	TTGGCCTTGCTGTCCTTG	82	(Monteiro et al., 2012)
	bla_{NDM} - R	ACACCAGTGACAATATCACCG		
Enterobacterales	bla_{CTXM} - F	ATGTGCAGYACCAGTAARGTKATGGC	590	(Trung at al., 2015)
	bla_{CTXM} - R	GGTRAARTARGTSACCAGAAYCAGCGG		
Pseudomonas aeruginosa	bla_{SPM} - F	CTAAATCGAGAGCCCTGCTTG	798	(Mendes, 2007)
	bla_{SPM} - R	CCTTTTCCGCGACCTTGATC		
Acinetobacter baumannii	bla_{OXA23}- F	GAT CGG ATT GGA GAA CCA GA	501	(Woodford et al., 2006)
	bla_{OXA23}- R	ATT TCT GAC CGC ATT TCC AT		

SOURCE: The author (2024).

SUPPLEMENTARY MATERIAL 2 - IDENTIFICATION USING MALDI-TOF-MS OF DRUG-RESISTANT

Codes Number	Species ID	Sample Date	Source	Organism (best macth)	Score[1]
CMRP6056	*Acinetobacter baumannii*	04/09/2016	Urine	*Acinetobacter baumannii*	2.38
CMRP6512	*Acinetobacter baumannii*	9/14/2016	Bone	*Acinetobacter baumannii*	2.40
CMRP6513	*Acinetobacter baumannii*	10/14/2016	Blood	*Acinetobacter baumannii*	2.54
CMRP6514	*Acinetobacter baumannii*	10/23/2016	Tracheal aspirate	*Acinetobacter baumannii*	2.54
CMRP6515	*Acinetobacter baumannii*	4/30/21	Blood	*Acinetobacter baumannii*	2.07
CMRP6516	*Acinetobacter baumannii*	4/30/21	Tracheal aspirate	*Acinetobacter baumannii*	2.36
CMRP6517	*Acinetobacter baumannii*	05/05/2021	Tracheal aspirate	*Acinetobacter baumannii*	2.39
CMRP6518	*Acinetobacter baumannii*	05/04/2021	Tracheal aspirate	*Acinetobacter baumannii*	2.39
CMRP6519	*Acinetobacter baumannii*	05/05/2021	Blood	*Acinetobacter baumannii*	2,37
CMRP6054	*Citrobacter freundii*	9/22/2016	Blood	*Citrobacter freundii*	2.47
CMRP6520	*Citrobacter freundii*	02/10/2016	Blood	*Citrobacter freundii*	2.37
CMRP6521	*Klebsiella aerogenes*	9/15/2016	Urine	*Klebsiella aerogenes*	2.36
CMRP6049	*Enterobacter cloacae*	10/13/2016	Blood	*Enterobacter cloacae*	2.11
CMRP6050	*Enterobacter cloacae*	02/10/2016	Blood	*Enterobacter cloacae*	2.36
CMRP6051	*Enterobacter cloacae*	05/09/2016	Blood	*Enterobacter cloacae*	2.35
CMRP6522	*Enterobacter cloacae*	05/09/2016	Rectal	*Enterobacter cloacae*	2.06
CMRP6523	*Enterobacter cloacae*	9/20/2016	Blood	*Enterobacter cloacae*	1.96
CMRP6524	*Enterobacter cloacae*	8/29/2016	Rectal	*Enterobacter cloacae*	2.26
CMRP6525	*Enterobacter cloacae*	9/30/2016	Urine	*Enterobacter cloacae*	2.32
CMRP6055	*Enterococcus faecium*	8/17/11	Rectal	*Enterococcus faecium*	2.18
CMRP6526	*Enterococcus faecium*	09/06/2011	Rectal	*Enterococcus faecium*	2.25

Codes Number	Species ID	Sample Date	Source	Organism (best macth)	Score[1]
CMRP6527	*Enterococcus faecium*	09/12/2011	Rectal	*Enterococcus faecium*	1.84
CMRP6528	*Enterococcus faecium*	9/26/11	Rectal	*Enterococcus faecium*	2.33
CMRP6529	*Enterococcus faecium*	01/02/2012	Rectal	*Enterococcus faecium*	2.21
CMRP6530	*Enterococcus faecium*	01/04/2012	Rectal	*Enterococcus faecium*	1.99
CMRP6531	*Enterococcus faecium*	1/16/12	Rectal	*Enterococcus faecium*	2.31
CMRP6532	*Enterococcus faecium*	1/18/12	Rectal	*Enterococcus faecium*	2.29
CMRP6533	*Enterococcus faecium*	02/07/2012	Rectal	*Enterococcus faecium*	1.99
CMRP6534	*Enterococcus faecium*	2/14/12	Rectal	*Enterococcus faecium*	2.02
CMRP6535	*Enterococcus faecium*	2/23/12	Rectal	*Enterococcus faecium*	2.13
CMRP6048	*Escherichia coli*	05/09/2016	Rectal	*Escherichia coli*	2.20
CMRP6536	*Escherichia coli*	04/09/2016	Urine	*Escherichia coli*	2.12
CMRP6537	*Escherichia coli*	05/09/2016	Rectal	*Escherichia coli*	2.23
CMRP6539	*Escherichia coli*	12/09/2016	Rectal	*Escherichia coli*	2.11
CMRP6540	*Escherichia coli*	9/20/2016	Blood	*Escherichia coli*	2.13
CMRP6541	*Escherichia coli*	9/21/2016	Urine	*Escherichia coli*	2.23
CMRP6542	*Klebsiella oxytoca*	02/09/2016	Secretion	*Klebsiella oxytoca*	2.09
CMRP6052	*Klebsiella pneumoniae*	05/09/2016	Rectal	*Klebsiella pneumoniae*	2.21
CMRP6053	*Klebsiella pneumoniae*	12/09/2016	Sputum	*Klebsiella pneumoniae*	1.99
CMRP6543	*Klebsiella pneumoniae*	9/18/2016	Urine	*Klebsiella pneumoniae*	2.32
CMRP6544	*Klebsiella pneumoniae*	9/13/2016	Blood	*Klebsiella pneumoniae*	1.82
CMRP6545	*Klebsiella pneumoniae*	9/13/2016	Urine	*Klebsiella pneumoniae*	2.48
CMRP6546	*Klebsiella pneumoniae*	9/16/2016	Urine	*Klebsiella pneumoniae*	1.82
CMRP6547	*Klebsiella pneumoniae*	9/16/2016	Urine	*Klebsiella pneumoniae*	2.08
CMRP6548	*Klebsiella pneumoniae*	9/19/2016	Rectal	*Klebsiella pneumoniae*	2.16
CMRP6549	*Klebsiella pneumoniae*	9/19/2016	Rectal	*Klebsiella pneumoniae*	2.37
CMRP6550	*Proteus mirabilis*	12/10/2016	Blood	*Proteus mirabilis*	2.34
CMRP6057	*Pseudomonas aeruginosa*	9/27/2016	Urine	*Pseudomonas aeruginosa*	2.46
CMRP6059	*Pseudomonas aeruginosa*	4/30/21	Tracheal aspirate	*Pseudomonas aeruginosa*	2.38
CMRP6551	*Pseudomonas aeruginosa*	10/22/2016	Secretion	*Pseudomonas aeruginosa*	2.37
CMRP6552	*Pseudomonas aeruginosa*	4/30/21	Blood	*Pseudomonas aeruginosa*	2.01
CMRP6553	*Pseudomonas aeruginosa*	10/11/2020	Blood	*Pseudomonas aeruginosa*	2.24
CMRP6554	*Pseudomonas aeruginosa*	12/03/2020	Tracheal aspirate	*Pseudomonas aeruginosa*	2.36
CMRP6058	*Staphylococcus aureus*	01/08/2021	Blood	*Staphylococcus aureus*	2.47
CMRP6555	*Staphylococcus aureus*	01/03/2021	Blood	*Staphylococcus aureus*	2.44
CMRP6556	*Staphylococcus aureus*	01/06/2021	Tracheal aspirate	*Staphylococcus aureus*	2.48
CMRP6557	*Staphylococcus aureus*	12/28/21	Biopsy	*Staphylococcus aureus*	2.36
CMRP6558	*Staphylococcus aureus*	12/30/21	Tracheal aspirate	*Staphylococcus aureus*	2.24
CMRP6559	*Staphylococcus aureus*	1/29/21	Blood	*Staphylococcus aureus*	2.45
CMRP6560	*Staphylococcus aureus*	07/03/2021	Tracheal aspirate	*Staphylococcus aureus*	2.43
CMRP6561	*Staphylococcus aureus*	7/17/21	Urine	*Staphylococcus aureus*	2.49
CMRP6562	*Staphylococcus aureus*	7/22/21	Biopsy	*Staphylococcus aureus*	2.33
CMRP6563	*Staphylococcus aureus*	7/24/21	Abscess	*Staphylococcus aureus*	2.36
CMRP6564	*Staphylococcus aureus*	7/26/21	Sputum	*Staphylococcus aureus*	2.50
CMRP6565	*Staphylococcus aureus*	08/07/2021	Tracheal aspirate	*Staphylococcus aureus*	2.37

SOURCE: The author (2024).

Notes:[1]Score - A score > 2.3 indicates “highly probable species identification”, a score > 2 and < 2.299 indicates “secure genus identification, probable species identification”, a score > 1.7 and < 1.999 indicates “probable genus identification”, and a score < 1.7 indicates “unreliable identification”.

SUPPLEMENTARY MATERIAL 3 - DISK-DIFFUSION SCREENING AND IDENTIFICATION OF RESISTANCE GENES BY *Polymerase chain reaction* (PCR)

Code Number*	Species ID	ETP	CIP	CPM	MER	GEN	CRO	AMI	AMP	VAN	S (300)	GE (30)	OXA	CLI	ERI	SUT	CAZ	IPM	Gene
CMRP6056	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*NDM
CMRP6512	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*OXA-23
CMRP6513	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*OXA-23
CMRP6514	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*OXA-23
CMRP6056	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*NDM
CMRP6512	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*OXA-23
CMRP6513	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*OXA-23
CMRP6514	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*OXA-23
CMRP6515	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*OXA-23
CMRP6516	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*OXA-23
CMRP6517	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*NDM
CMRP6518	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*OXA-23
CMRP6519	*A. baumannii*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	R	R	*bla*OXA-23
CMRP6054	*C. freundii*	-	R	R	S	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*CTX-M
CMRP6520	*C. freundii*	-	R	R	S	S	R	R	-	-	-	-	-	-	-	-	-	-	*bla*CTX-M
CMRP6521	*K. aerogenes*	-	R	R	R	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*NDM
CMRP6049	*Enterobacter cloacae*	R	S	R	S	S	R	-	-	-	-	-	-	-	-	-	-	-	*bla*KPC
CMRP6050	*Enterobacter cloacae*	R	R	R	S	R	R	-	-	-	-	-	-	-	-	-	-	-	None
CMRP6051	*Enterobacter cloacae*	R	R	R	R	R	R	-	-	-	-	-	-	-	-	-	-	-	*bla*KPC
CMRP6522	*Enterobacter cloacae*	S	R	R	S	R	R	-	-	-	-	-	-	-	-	-	-	-	*bla*CTX-M

CMRP6523	*Enterobacter cloacae*	S	R	R	S	R	R	-	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6524	*Enterobacter cloacae*	R	R	R	R	R	R	-	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6525	*Enterobacter cloacae*	R	R	R	R	R	R	-	-	-	-	-	-	-	-	-	-	-	*bla*$_{NDM}$
CMRP6055	*E. faecium*	-	R	-	-	-	-	-	R	R	R	R	-	-	-	-	-	-	*bla*$_{NDM}$
CMRP6526	*E. faecium*	-	R	-	-	-	-	-	R	R	R	R	-	-	-	-	-	-	*vanA*
CMRP6527	*E. faecium*	-	R	-	-	-	-	-	R	R	R	R	-	-	-	-	-	-	*vanA*
CMRP6528	*E. faecium*	-	R	-	-	-	-	-	R	R	R	R	-	-	-	-	-	-	*vanA*
CMRP6529	*E. faecium*	-	R	-	-	-	-	-	R	R	R	R	-	-	-	-	-	-	*vanA*
CMRP6530	*E. faecium*	-	R	-	-	-	-	-	R	R	R	R	-	-	-	-	-	-	*vanA*
CMRP6531	*E. faecium*	-	R	-	-	-	-	-	R	R	R	R	-	-	-	-	-	-	*vanA*
CMRP6532	*E. faecium*	-	R	-	-	-	-	-	R	R	R	R	-	-	-	-	-	-	*vanA*
CMRP6533	*E. faecium*	-	R	-	-	-	-	-	R	R	R	R	-	-	-	-	-	-	*vanA*
CMRP6534	*E. faecium*	-	R	-	-	-	-	-	R	R	R	R	-	-	-	-	-	-	*vanA*
CMRP6535	*E. faecium*	-	R	-	-	-	-	-	R	R	R	R	-	-	-	-	-	-	*vanA*
CMRP6048	*E. coli*	-	R	R	S	R	R	R	-	-	-	-	-	-	-	-	-	-	None
CMRP6536	*E. coli*	-	S	R	S	S	R	-	-	-	-	-	-	-	-	-	-	-	None
CMRP6537	*E. coli*	-	R	R	S	R	R	-	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6539	*E. coli*	-	R	R	S	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6540	*E. coli*	-	S	S	S	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6541	*E. coli*	-	R	R	S	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6542	*K. oxytoca*	-	R	R	S	S	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6052	*K. pneumoniae*	-	R	R	S	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6053	*K. pneumoniae*	-	R	R	R	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6543	*K. pneumoniae*	-	R	R	S	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{NDM}$
CMRP6544	*K. pneumoniae*	-	S	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-	*bla*$_{NDM}$

CMRP6545	*K. pneumoniae*	-	R	R	S	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6546	*K. pneumoniae*	-	R	R	S	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6547	*K. pneumoniae*	-	R	R	R	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6548	*K. pneumoniae*	-	R	R	R	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6549	*K. pneumoniae*	-	R	R	R	R	R	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6550	*Proteus mirabilis*	-	S	R	S	S	S	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{CTX-M}$
CMRP6057	*P. aeruginosa*	-	R	R	S	R	-	R	-	-	-	-	-	-	-	-	-	-	*bla*$_{KPC}$
CMRP6059	*P. aeruginosa*	-	R	R	R	R	-	S	-	-	-	-	-	-	-	-	R	R	*bla*$_{SPM}$
CMRP6551	*P. aeruginosa*	-	R	R	R	R	-	R	-	-	-	-	-	-	-	-	-	-	None
CMRP6553	*P. aeruginosa*	-	R	R	R	R	-	S	-	-	-	-	-	-	-	-	R	R	*bla*$_{NDM}$
CMRP6554	*P. aeruginosa*	-	R	S	R	R	-	S	-	-	-	-	-	-	-	-	R	R	*bla*$_{NDM}$
CMRP6555	*S. aureus*	-	R	-	-	S	-	-	-	-	-	-	R	R	R	S	-	-	*mec*A
CMRP6556	*S. aureus*	-	R	-	-	S	-	-	-	-	-	-	R	R	R	R	-	-	*mec*A
CMRP6557	*S. aureus*	-	R	-	-	S	-	-	-	-	-	-	R	S	R	S	-	-	*mec*A
CMRP6558	*S. aureus*	-	R	-	-	S	-	-	-	-	-	-	R	R	R	S	-	-	*mec*A
CMRP6559	*S. aureus*	-	S	-	-	S	-	-	-	-	-	-	R	R	R	S	-	-	*mec*A
CMRP6560	*S. aureus*	-	R	-	-	S	-	-	-	-	-	-	R	R	R	S	-	-	*mec*A
CMRP6561	*S. aureus*	-	R	-	-	R	-	-	-	-	-	-	R	R	R	R	-	-	*mec*A
CMRP6562	*S. aureus*	-	R	-	-	S	-	-	-	-	-	-	R	R	R	S	-	-	*mec*A
CMRP6563	*S. aureus*	-	R	-	-	S	-	-	-	-	-	-	R	R	R	S	-	-	*mec*A
CMRP6564	*S. aureus*	-	R	-	-	S	-	-	-	-	-	-	R	R	R	S	-	-	*mec*A
CMRP6565	*S. aureus*	-	R	-	-	R	-	-	-	-	-	-	R	R	R	R	-	-	*mec*A

SOURCE: The author (2024).

Legend: ETP-Ertapenen; CIP-Ciprofloxacin; CPM- Cefepime; MER- Meropenen; GEN – Gentamicin; CRO Cefuroxime; AMI- Amikacin; AMP- Ampicilian; VAN- Vancomycin; S (300) - Streptomycin; CN (30) -high-concentration gentamicin; OXA- Oxacillin; CLI- Clindamycin;ERI- Erythromycin; SUT-Trimethoprim-sulfamethoxazole; CAZ- Ceftazidime; IPM-Imipenem. R-Resistant; S-Sensible; (-): not evaluated in this study. *CMRP- Microbiological Collections Centre of the Taxonline Network of Paraná (CMRP/Taxonline)

4 CHAPTER III

Streptomyces spp. ALCOHOLIC EXTRACTS FROM MARINE SEDIMENTS WITH ANTIMICROBIAL ACTIVITY AGAINST MULTIDRUG RESISTANT BACTERIA

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4.1 ABSTRACT

This study in vitro evaluated the antimicrobial activity of *Streptomyces* spp isolated from marine sediments on Ilha do Mel, Paraná, Brazil. The bacterial extracts were previously shown to exhibit antimicrobial activity against pathogenic strains. In the context of the global threat of antimicrobial resistance (AMR), there is an urgent need for the discovery of new bioactive secondary metabolites with antibacterial properties. The objective was to assess the antimicrobial activity of four *Streptomyces* strains against twelve bacteria resistant to various drugs, including Gram-positive and Gram-negative pathogenic strains with different resistance mechanisms. The minimum inhibitory concentration (MIC) value of ≥62.5µg/mL indicated the antibiotic activity of *Streptomyces cavourensis* CMRP6046 against vancomycin-resistant *Enterococcus faecium*. The *in vitro* analysis of the alcoholic extract indicated that the compounds produced by *S. cavourensis* CMRP6046 that inhibit *Enterococcus faecium* began to act after 96 hours of incubation, coinciding with the peak in biomass production. Furthermore, bioguided fractionation of the crude alcoholic extract of *S. cavourensis* CMRP6046 demonstrated that three (F1, F2, and F4) of the seven fractions tested exhibited inhibition rates above 50%. Therefore, this strain has been identified as a promising natural source for the production and isolation of secondary metabolites with potential applications in the pharmaceutical industry. Future studies will focus on the fractionation and purification of bioactive compounds and the quantitative determination of safe concentrations that can be used to enhance existing drugs or to obtain new bioproducts against multidrug resistant bacteria.

Keywords: *Streptomyces*; antibacterial resistance; secondary metabolites.

4.2 INTRODUCTION

Nature is considered the primary source of new biotechnologically relevant molecules with applications in different fields. The production of natural products by microorganisms is largely associated with the *Actinobacteria phylum*, which is

endowed with rich metabolic machinery that enables the production of bioactive compounds (Ajar Nath, 2021). *Streptomyces* is the main genus of the actinobacteria phylum, where most secondary metabolite-producing bacteria are found (Alam et al., 2022).

The demand for new natural products has led to a recent focus on the marine environment. In fact, ocean bioprospecting has opened prospects for the discovery of new drugs and other useful chemical structures due to the enormous unexplored biodiversity found in these environments (Rusyaev; Orlov, 2024).

Microbial secondary metabolites, i.e., antibiotics, pigments, growth hormones, antitumor agents, and others, are not essential for the growth and development of microorganisms but have shown great potential for human and animal health. Antibiotics such as streptomycin, gentamicin, rifampicin, chloramphenicol, and erythromycin are produced by *Streptomyces* spp. (Al-ShaibaniI et al., 2021). These bioactive compounds are mainly produced by the activation of cryptic gene clusters that are not active under normal conditions and, therefore, the expression of these clusters would be useful to explore the chemical diversity of microorganisms (Atanasov et al., 2021).

Antimicrobial resistance (AMR) is an increasingly worrying global public health concern, the microorganisms are acquiring resistance, which makes it difficult for antibiotics to fight infections and ineffective. Multidrug resistance (MDR) in bacteria occurs through the accumulation, on plasmids or resistance transposons (R), each encoding resistance to a specific agent and/or through the action of multidrug efflux pumps, each of which can pump out more than one type of drug (De Gaetano et al., 2023).

In 2017, the WHO published a list of "priority pathogens" resistant to antibiotics. The list was drawn up to guide and promote research and development. The WHO reported very high rates of resistance in bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Staphylococcus aureus*, considering that many of these organisms exhibit simultaneous resistance to multiple antimicrobials, a phenotype that is referred to as multidrug resistance (MDR) and can be attributable to single or multiple resistance mechanisms, representing a typical challenge for treatment (WHO, 2017).

Therefore, to struggle with the development of resistance and to have effective antibiotics available, constant efforts are needed to discover and improve antibiotics

(CLSI, 2019). Most current drugs to treat bacterial infections are natural products and their derivatives, so natural products are an abundant source of new biologically active molecules, making them the mainstay of modern medicine and essential for human health (Elmaidomy et al., 2022).

In bioprospecting studies of microorganisms isolated from the intertidal region of Ilha do Mel, Paraná, Brazil, around 60 fungi and 116 actinobacteria from the genera *Nocardia* and *Streptomyces* were found to have inhibitory activities against the pathogenic reference strains *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231) (Porsani et al., 2013; Porsani et al., 2017).

Given the need to search for new antibiotics, the aim of this study was to bioprospect some strains of the genus *Streptomyces* isolated from the intertidal region of Ilha do Mel, Paraná, which previous studies (Porsani et al., 2013; Porsani et al., 2017) have shown to be promising in terms of their antimicrobial potential against pathogenic reference bacteria. However, for the current study, they were tested against MDR bacteria from a tertiary hospital and belonging to the Microbiological Collections Centre of the Taxonline Network of Paraná (CMRP/Taxonline), in addition to characterizing the bioactive compounds capable of inhibiting the growth of these multidrug-resistant bacteria.

4.3 MATERIALS AND METHODS

4.3.1 Biological Samples

A total of four *Streptomyces* genus strains isolated from the intertidal region of Ilha do Mel, Paraná, previously identified and characterized by Porsani et al (2013), were used to evaluate their antibacterial potential (Table 4).

TABLE 4 - STRAINS THE *Streptomyces* spp. ISOLATED FROM THE INTERTIDAL REGION OF ILHA DO MEL WITH ANTIMICROBIAL ACTIVITY

Strains	Code[1]	Source	GenBank
Streptomyces sp.	CMRP6044	Marine sediment	-
Streptomyces cavourensis	CMRP6047	Marine sediment	JX997143
Streptomyces cavourensis	CMRP6046	Marine sediment	JX997144
Streptomyces bacillaris	CMRP6045	Marine sediment	JX997140

SOURCE: The author (2024).

Notes: [1] Microbiological Collections Centre of the Taxonline Network of Paraná (CMRP/Taxonline).

In addition, twelve multi-drug resistant (MDR) bacterial clinical strains from the Clinical Hospital Complex of the Federal University of Parana (CHC/UFPR) (Table 5).

TABLE 5 - BACTERIAL CLINICAL STRAINS FROM THE CLINICAL HOSPITAL COMPLEX OF THE FEDERAL UNIVERSITY OF PARANA EVALUATED

Strains	Code[5]	Source	Acquired Resistance
Escherichia coli	CMRP6048	Rectal swab	RGC[1]
Enterobacter cloacae	CMRP6049	Blood	CR[2]
Enterobacter cloacae	CMRP6050	Blood	RGC
Enterobacter cloacae	CMRP6051	Blood	CR
Klebsiella pneumoniae	CMRP6052	Rectal swab	RGC
Klebsiella pneumoniae	CMRP6053	Sputum	CR
Citrobacter freundii	CMRP6054	Blood	RGC
Enterococcus faecium	CMRP6055	Rectal swab	VRE[3]
Acinetobacter baumannii	CMRP6056	Urine	CR
Pseudomonas aeruginosa	CMRP6057	Blood	CR
Staphylococcus aureus	CMRP6058	Blood	MRSA[4]
Pseudomonas aeruginosa	CMRP6059	Tracheal aspirate	CR

SOURCE: The author (2024).

Legend: [1] Resistant to third-generation cephalosporins; [2]Carbapenem-resistant; [3]Vancomycin-resistant Enterococcus; [4]Methicillin-resistant *S. aureus*; [5]Microbiological Collections of the Taxonline Network of the Paraná.

4.3.2 Antimicrobial activity screening

4.3.2.1 Preparation of cell-free filtrate (CFF) of *Streptomyces* spp.

Streptomyces spp. (Table 4) was grown for five days on GYA (glucose and yeast extract agar) plates, pH 5.8 at 28ºC. Two 3 mm plugs were inoculated into 50mL of GYB (glucose and yeast extract broth) for 3 days and cultivated under orbital shaking (150 rpm, 28ºC). After this period, a volume of 1mL pre-cultivation was inoculated into an Erlenmeyer flask (250mL) containing 99mL of GYB and cultivated under orbital shaking (150 rpm, 28ºC) for 7 days. The mycelium was separated from the culture medium by filtration with Whatman n◦4 filter paper (Gos et al., 2017). The CFF was stored at -20°C in a freezer for later evaluation in the liquid antagonism test.

The pathogenic bacteria strains (Table 5) used to evaluate the antibacterial activity of the *Streptomyce*s sp. strains were inoculated into Erlenmeyer flask (125mL) containing 50mL TSB (Tryptone Soy Broth) medium, with the aid of an inoculation loop and incubated at 36±1◦C under orbital shaking (120 rpm) until the OD600 eached 0.2, measured by a spectrophotometer (λ = 600 nm).

To evaluate the antimicrobial activity, a volume of 100µL aliquots of the standardized inocula of pathogenic bacterial strains were distributed into 96 wells of microdilution plates according to Silva et al (2018) with adaptations. Additionally, 100µL of CFF from each *Streptomyces* sp. strain was added to each well (3 wells for each strain tested). To serve as negative controls, three wells were prepared: one containing 100µL of l GYB and 100µL of TSB, one containing 100µL of CFF and 100µL of TSB, and one containing 100µL of the standardized inocula of pathogenic bacterial strains 100µL of GYB. Ciprofloxacin (8µg/mL) was employed as a positive control. The plates were incubated for 48 hours at 37°C, with absorbance readings (λ = 600 nm) taken at 24 and 48 hours. This procedure was conducted in triplicate. Data were collected on a microplate reader (Themo Scientific™ Multiskan Sky 260) set to OD600 (bacterial inhibition assay). The results were expressed as percentage inhibition (%), which represented the difference in the average absorbance of each treatment with the control growth (Gudiña et al., 2010).

4.3.2.2 Obtaining the alcoholic extract of strain *Streptomyces* spp.

Streptomyces spp. were grown for seven days on GYA plates, pH 5.8 at 28◦C. The two plug 3mm of the isolate were inoculated into 50 mL of GYB for 3 days. After this period, a volume of 1mL of the pre-culture was inoculated into Erlenmeyer flasks (250mL) containing 99mL of GYB and cultivated under orbital agitation (150 rpm, 28◦C) for 7 days. The mycelium was separated from the culture medium by filtration with Whatman n◦4 filter paper (Gos et al., 2017). The fermentation liquid was mixed with 1% (w/v) XAD-16 resin and stirred overnight (150 rpm, 28◦C), followed by centrifugation. The resin was washed with water and methanol was added to extract the metabolites. The MeOH extract obtained was dried using a rotary evaporator at 40◦C and then weighed (Iantas, J. et al., 2021).

4.3.2.3 Minimum Inhibitory Concentration (MIC) alcoholic extract of the *Streptomyces cavourensis* CMRP6046

The *S. cavourensis* CMRP6046 extract was subjected to a microdilution assay following the EUCAST methodology and the modified protocol previously described by Iantas et al. (2021). First, the stock solution was diluted in Mueller-Hinton broth (MHB)

containing 1% dimethyl sulfoxide (DMSO v/v) to a final concentration of 10mg/mL. This solution was then inoculated (50µL volume) in decreasing dilutions in triplicate, starting with 1 mg/mL (for a 200µL final volume), in a 96-well plate. This procedure was conducted for bacterial pathogens. A volume of 20 µL aliquot of pathogens bacterial cultures reactivated to a concentration of 10^8 CFU/mL was added to each well, except for the blank (negative control). The negative control consisted of 200 µL of MHB. Ciprofloxacin (8µg/L) was employed as a test control. The vehicle control consisted of a solution of 1% (v/v) DMSO in MHB medium. The plates were incubated for 48 hours at 36±1°C, and the antimicrobial activity was quantified at 24 hours intervals by spectrophotometry using a Thermo Scientific™ Multiskan Sky 260 microplate spectrophotometer calibrated to OD600.

According to the methodology previously described by Elshikh et al. (2017), the minimum bactericidal concentration (MBC) was determined by directly plating 10 µL of the contents of the wells with concentrations equal to and greater than the MIC value. In the absence of colony growth or a maximum of three colony-forming units (CFU), the extract was classified as bactericidal. If the number of CFU exceeded 3, the bacterial extract was considered bacteriostatic. Three independent experiments were carried out in triplicate according to CLSI guidelines (2020). The EUCAST 2023 clinical breakpoints were applied to ciprofloxacin to determine the appropriate course of action. The following control strains were used in this study: *E. coli* ATCC 25922*, S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853 and *E. faecalis* ATCC 29212 (EUCAST, 2023).

4.3.2.4 Estimation of growth by determination of dry biomass weight and production of antibacterial agents of *Streptomyces cavourensis* CMRP6046

The preparation of the CFF of *S. cavourensis* CMRP6046 was obtained according to the methodology described in item 3.2.2.1.1. Subsequently, the filter paper containing the biomass was subjected to an oven treatment at 50 °C for a period of overnight, with the objective of stabilizing the weight of the sample. (Ghashghaei, S. et al., 2018). A processed at intervals of 16, 24, 40, 48, 72, 96, 120, 144, 168, 192, 216 and 244 hours of incubation.

4.3.2.5 Thin-layer chromatography (TLC) of alcoholic extract of *Streptomyces cavourensis* CMRP6046

The TLC of alcoholic extract of *S. cavourensis* CMRP6046 was carried out on a TCL plate for the dye's methyl orange and methylene blue, which contains silica gel 60 with 0.2 mm thick particles as the stationary phase. The elution of the compounds was monitored with the aid of developers. The chemical solvents that were used as developers for the organic compounds were potassium hydroxide, aluminum chloride, ferric chloride, vanillin, phosphomolybdic acid, anisaldehyde solution, Dragendorf solution and ethanolic sulfuric acids H2SO4 solution (5% v/v).

4.3.2.6 Bioguided fractionation of the alcoholic extract of *Streptomyces cavourensis* CMRP6046

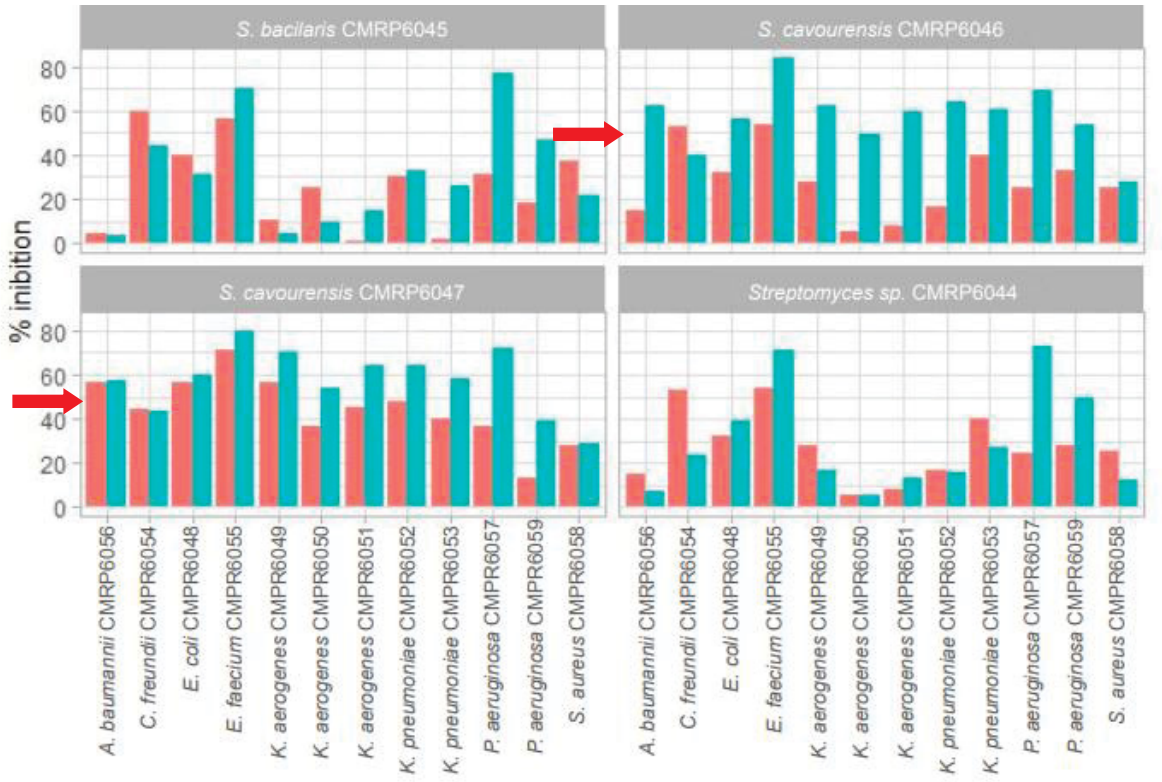
A sample of flash silica weighing approximately 58.3 grams was placed in a vacuum column. The column was then solubilized, and the sample was applied to the silica surface (a piece of cotton was placed on top) to prevent the solvent from moving the silica. Subsequently, a series of solvent mixtures were employed to generate fractions with varying polarities, spanning from the most apolar to the most polar (Snyder and Dolan, 2006; Snyder et al., 2009). The fractions were then subjected to analysis to ascertain the inhibition rate against the pathogenic strain *E. faecium* CMRP6055, in accordance with the methodology delineated in item 4.3.2.

4.4 RESULTS

4.4.1 Screening the antimicrobial activity of *Streptomyces* spp.

The four strains of *Streptomyces* spp. showed antimicrobial activity, as evidenced by their bacterial inhibition rates of over 50% in the screening tests carried out against pathogenic bacteria, according to the methodology detailed in Section 4.3.2. However, only strains CMRP6046 and CMRP6047 were subjected to the production of an alcoholic extract. Both showed bacterial inhibition rates of over 50% against nine of the twelve multidrug resistant bacterial strains (Figure 5 and S.M. 4).

FIGURE 5 - RESULTS OF SCREENING THE ANTIMICROBIAL ACTIVITY OF *Streptomyces* spp.



SOURCE: The author (2024).

Notes: CMRP/Taxoline - Microbiological Collections of the Taxonline Network of the Paraná.

4.4.2 Obtaining the alcoholic extract and Minimum Inhibitory Concentration (MIC) of strain *Streptomyces cavourensis* CMRP6046

The *Streptomyces* strains CMRP6046 and CMRP6047 yielded alcoholic extracts with dry weights of 180 mg and 95 mg, respectively. Therefore, CMRP6046 was selected for the determination of the MIC and for the subsequent analyses, given its higher dry extract weight. The CMRP6046 strain produced an extract with bioactivity against the *Enterococcus faecium* strain CMRP6055 (resistant to vancomycin), with a minimum inhibitory concentration of ≥62.5µg/mL (Table 6).

TABLE 6 - MINIMUM INHIBITORY CONCENTRATION OF THE ALCOHOLIC EXTRACT OF *Streptomyces cavourensis* CMRP6046

Species	Code Number*	MIC value	MBC[1]
Escherichia coli	CMRP6048	≥ 500 µg/mL	BS[2]
Enterobacter cloacae	CMRP6049	≥ 1000 µg/mL	BS
Enterobacter cloacae	CMRP6050	≥ 1000 µg/mL	BS
Enterobacter cloacae	CMRP6051	≥ 1000 µg/mL	BS
Klebsiella pneumoniae	CMRP6052	≥ 1000 µg/mL	BS
Klebsiella pneumoniae	CMRP6053	≥ 1000 µg/mL	BS
Citrobacter freundii	CMRP6054	≥ 1000 µg/mL	BS
Enterococcus faecium	CMRP6055	≥ 62.5 µg/mL	BC[3]
Acinetobacter baumannii	CMRP6056	≥ 500 µg/mL	BC
Pseudomonas aeruginosa	CMRP6057	≥ 1000 µg/mL	BS
Staphylococcus aureus	CMRP6058	≥ 1000µg/mL	BS
Pseudomonas aeruginosa	CMRP6059	≥ 1000µg/mL	BS

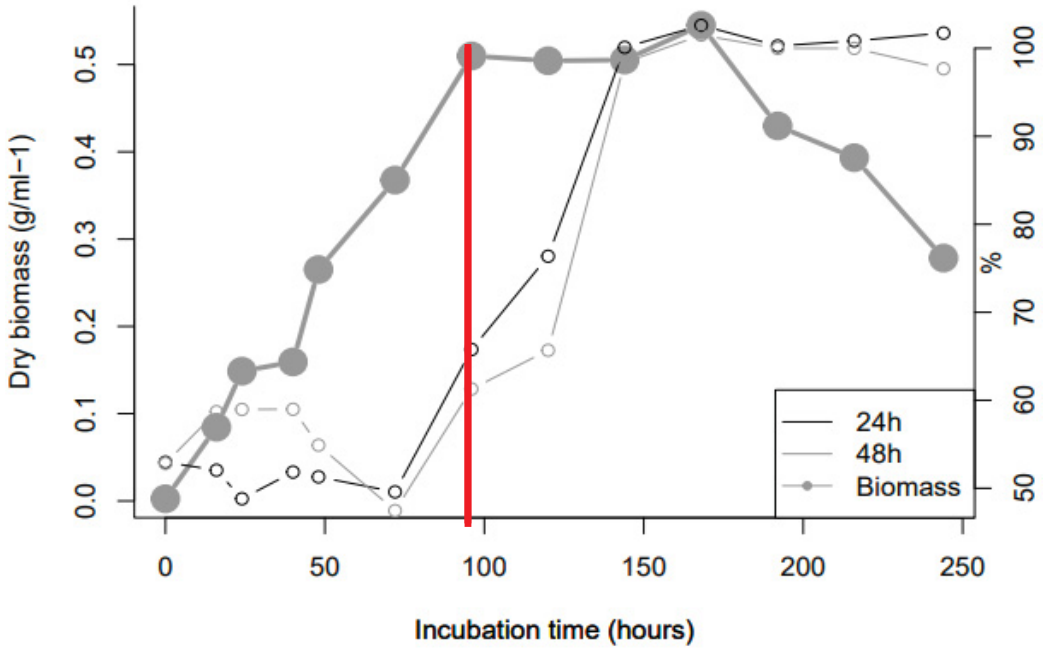
SOURCE: The author (2024).

Legend: [1]MBC - Minimum bactericidal concentration ;[2]BS - bacteriostic; [3]BC- bactericidal.
*Microbiological Collections of the Taxonline Network of the Paraná

4.4.3 The complex relationship between antibacterial agent production and in vitro growth of the *Streptomyces cavourensis* CMRP6046

S. cavourensis CMRP6046 exhibited an exponential increase in its growth curve obtained through dry biomass weight after 16 hours of incubation, which persisted until 96 hours, accompanied by a reduction from 168 hours onwards. This represents the most crucial phase of bacterial growth, as the expression of the biosynthetic gene cluster of the antibacterial agent(s) was at detectable levels just after 96h of incubation, during the middle of the log phase. Subsequently, the expression of the antibacterial agent(s) reached its highest level at the end of the first descending phase (168h). After that, expression remained stable until 244h (Figure 6). Furthermore, it was possible to observe that the expression of the antibacterial agent(s) was similar at 24 and 48 hours.

FIGURE 6 - ESTIMATION OF GROWTH BY DETERMINATION OF DRY BIOMASS WEIGHT AND PRODUCTION OF ANTIBACTERIAL AGENT(S) OF THE *Streptomyces cavourensis* CMRP6046 WITHIN ABOUT 24 AND 48 HOURS



SOURCE: The author (2024).

4.4.4 Thin-layer chromatography (TLC) and Bioguided fractionation of the alcoholic extract of *Streptomyces cavourensis* CMR6046

After several tests to find the best mobile phase for the TLC, the one with the best results was DCM (Dichloromethane)/MeOH (Methanol) 85:15. The TLC of the bacterial methanolic extract of *S. cavourensis* CMRP6046 revealed a maximum of eight classes of organic compounds (Table 7).

TABLE 7 - THIN LAYER CHROMATOGRAPHY (TLC) PROFILE OF THE ALCOHOLIC EXTRACT OF *Streptomyces. cavourensis* CMRP6046

Solvents	Detection[1]	Results
Potassium hydroxide	Coumarins, anthrones, and anthraquinone;	Positive
Dragendorf solution	Alkaloids;	Negative
Anisaldehyde solution	Steroids and terpenoids;	Positive
Aluminum chloride	Flavonoids;	Positive
Ethanol solution of H_2SO^4 5%	Universal reactive	Positive
Iron chloride	Phenols and flavonoids;	Positive
Vanillin	Steroids, sensitive terpenoids and flavonoids to alcohols;	Positive
Phosphomolybdic acid	For general use	Positive

SOURCE: The author (2024).

Note: [1]Detection classes of organic compounds.

The alcoholic extract of *S. cavourensis* CMRP6046 was fractionated according to the sequence of the solvent system in a gradient of increasing polarity. This process led to the isolation of eight distinct fractions, which are detailed in Table 8.

TABLE 8 - THE FRACTIONATION OF THE ALCOHOLIC EXTRACT OF *Streptomyces cavourensis* CMRP6046 ACCORDING TO THE SEQUENCE OF THE SOLVENT SYSTEM IN AN INCREASING POLARITY GRADIENT

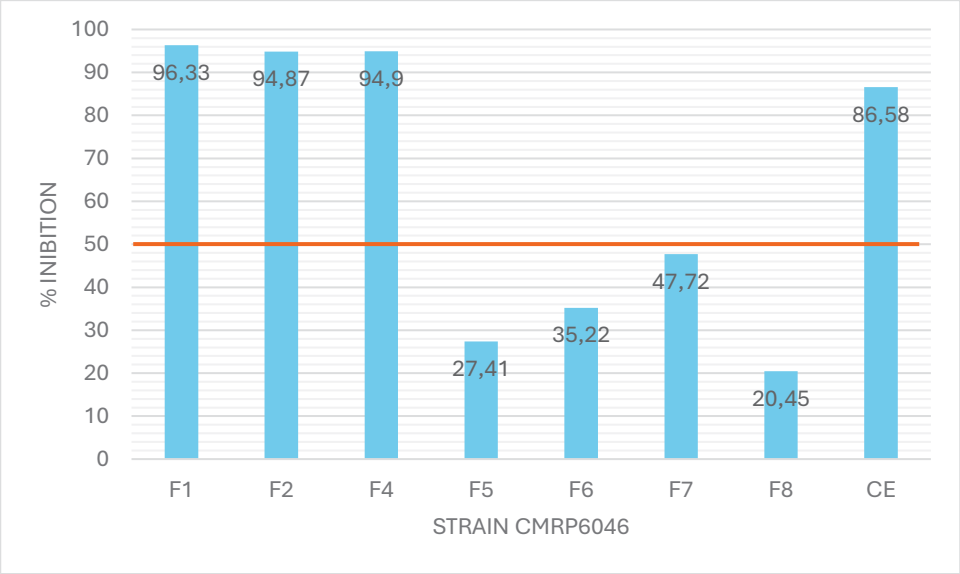
Fractions	Solvent Systems	Masses mg/mL[1]
F1	Dichloromethane/Hexane 1:1 (400 mL)	33
F2	Dichloromethane 100% (100 mL)	30
F3*	Dichloromethane/Methanol 80:20 (250mL)	*
F4	Dichloromethane/Methanol 60:40 (300mL)	46
F5	Dichloromethane/Methanol 60:40 (300mL)	29
F6	Dichloromethane/Methanol 40:60 (300mL)	35
F7	Dichloromethane/Methanol 20:80 (300mL)	29
F8	Methanol 100% (300 mL.)	41

SOURCE: The author (2024).

Notes: * It was not possible to conduct a test of the F3 due to the complete evaporation of the substance. **The molecular masses of the fractions obtained.

Following fractionation, the fractions were subjected to analysis of bacterial activity against the multidrug resistant *Enterococcus faecium* strain CMRP6055. Fractions F1, F2, and F4 may warrant further analysis given their observed inhibition rates of 96.33%, 94.87%, and 94.90%, respectively. These rates were found to be slightly higher than that of the crude extract, with an observed inhibition rate of 86.58% (Figure 7).

FIGURE 7 - INHIBITORY EFFECTS OF THE *Streptomyces cavourensis* CMRP6046 OF THE FRACTIONS ALCOOLIC EXTRACTS AGAINST THE *Enterococcus faecium* CMRP6055



SOURCE: The author (2024).

Legend: Positive result % inhibition rate of bacterial growth above 50%; F-Fraction; CE- crude extract.

4.5 DISCUSSION

This study analyzed actinobacteria of the genus *Streptomyces* associated with marine sediments collected from the intertidal region of Ilha do Mel, Paraná, Brazil (Porsani, M. V. et al., 2013). In previous studies, the species selected for this study have already been found to have antimicrobial activity against relevant pathogenic strains (*S.aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *C. albicans* ATCC 10231) (Porsani et al., 2013). In view of this, the objective of the current research was to evaluate their potential to inhibit bacteria resistant to multiple drugs, since AMR is a worldwide problem and there is an urgent need to contribute by providing new bioactive secondary metabolites with antibacterial capacity.

According to MIC values (Table 6), the *S. cavourensis* CMRP6046 alcoholic crude extract of showed the best antibiotic activity against the pathogenic strain of vancomycin-resistant *Enterococcus faecium* (VRE) CMRP6055. This result is corroborated by reports in the literature describing extracts obtained from this genus isolated from marine environments with diverse biological activities, including antibacterial activities against resistant pathogenic bacteria (Clavo et al., 2021; Sabido et al., 2021).

The crude extract of *S. cavourensis* strain CMRP6046 inhibited the growth of the VRE CMRP6055 with MIC value 62.5µg/mL (Table 6). However, compared to other reports, the MIC result was higher than that observed in this study, for example, the crude alcoholic extract of *Streptomyces lienomycini* isolated from soil in El-Bahariya Oasis (Egypt) obtained a MIC value of 125 µl/mL *E. faecium* TS7 (Elsayed et al., 2022). The least hit rate of 13% that is, 2 isolates out of a total of 15 *S. cavourensis* isolated from the marine sediments of Visayan Sea, Philippines was observed against VRE (Sabido et al., 2021), a leading cause of hospital acquired infection (Zhou et al., 2020). analysis of an antimicrobial extract prepared from culture broth of the marine-derived actinomycete *S. cavourensis*. G278 showed that a compound was found to have antibacterial activity against *E. faecalis* (MIC: 256 µg/mL) (Cao et al.,2021).

The *S. cavourensis* CMRP6046 was active against the Gram-negative members of ESKAPEE pathogens (*E. coli*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae*. however, the MIC values were relatively high (1000µg/mL to 500µg/mL). The low hit rate is due to the complex outer membrane and efflux pumps of gram-negative bacteria, which change how drugs bind to cells and how much gets inside (Gaurav et al., 2023).

The relationship between the reduction of antibacterial agent and the *in vitro* growth of *S. cavourensis* CMRP6046 through biomass production (Figure 6), it was possible to observe that compounds with antibacterial activity against VRE began to inhibit at 96 hours of incubation of the fermentation broth, which reinforces the results of the curve of growth of the *S. cavourensis* CMRP6046 strains analyzed through biomass production, which reached its maximum peak at 96 hours of experiment. Therefore, these results corroborate that most antibiotics and other secondary metabolites are indeed produced during the idiophase, or stationary phase, of microbial growth (Wang et al., 2022). This is considered a general characteristic of secondary metabolite fermentations, as opposed to primary metabolites which are produced during the trophophase or growth phase (Ruiz-Villafan et al., 2022).

The results presented in this work so far have been promising and could be extremely important in helping to treat pathologies caused by bacterial agents, as they are included in the pathogenic group known as "ESKAPEE", which includes the pathogens: *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa,* species of the genus *Enterobacter* and *E. coli*.

In depth chromatographic analyses of the *S. cavourensis* CMRP6046 secondary metabolites belonging to the classes of compounds: flavonoids, phenolic acids, alkaloids, terpenoids and others, are underway. Future studies are aimed at the fractionation and purification of bioactive compounds and the quantitative determination of safe concentrations that can be used to improve existing drugs or to obtain new bioproducts against MDR bacteria.

CONFLICT OF INTERESTS

The authors declare no competing interest.

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SUPPLEMENTARY MATERIAL 4 - SCREENING RESULTS FOR THE ANTIMICROBIAL ACTIVITY OF S*treptomyces* spp. AGAINST MULTIDRUGS RESISTANT BACTERIA WITHIN ABOUT 24 AND 48

	***Streptomyces* sp. CMRP6044**		***S. bacilaris* CMRP6045**		***S. cavourensis* CMRP6046**		***S. cavourensis* CMRP6047**	
	24h	**48h**	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**
***E. coli* CMRP6048**	**32.15**	**38.80**	**39.86**	**31.13**	**32.15**	**56.69[1]**	**56.73[1]**	**60.29[1]**
***E. cloacae* CMRP6049**	27.94	16.16	10.36	4.62	27.94	**62.32[1]**	**56,86[1]**	**70.78[1]**
***E. cloacae* CMRP6050**	5.28	5.05	25.22	9.38	5.28	**49.62[1]**	36.59	**54.23[1]**
***E. cloacae* CMRP6051**	7.56	13.28	1.29	14.80	7.56	**59.76[1]**	44.99	**64.65[1]**
***K. pneumoniae* CMRP6052**	16.45	15.59	30.11	32.84	16.45	**64.15[1]**	47.74	**64.34[1]**
***K. pneumoniae* CMRP6053**	40.00	26.61	2.09	25.92	40.00	**60.57[1]**	39.72	**58.35[1]**
***C. freundii* CMRP6054**	**53.36[1]**	23.20	**59.63[1]**	44.55	**53.36[1]**	40.29	43.97	43.68
***E. faecium* CMRP6055**	**53.76[1]**	**71.20[1]**	**56.47[1]**	**70.79[1]**	**53.76[1]**	**84.54[1]**	**71.20[1]**	**79.99[1]**
***A. baumannii* CMRP6056**	14.91	7.18	4.52	3.08	14.91	**62.91[1]**	**56.69[1]**	**57.82[1]**
***P. aeruginosa* CMRP6057**	24.80	**73.3[1]**	31.56	**77.17[1]**	24.80	**69.53[1]**	36.36	**72,43[1]**
***S. aureus* CMRP6058**	25.44	12.35	37.47	21.59	25.44	27.78	27.70	28.39
***P. aeruginosa* CMRP6059**	27.61	49.54	18.22	46.92	32.68	**54.16[1]**	13.10	39.50

SOURCE: The author (2024).

Legend:[1]Positive result % inhibition rate of bacterial growth above 50%; CMRP- Microbiological Collections of the Taxonline Network of the Paraná.

5 CHAPTER IV

IDENTIFICATION OF GENES CLUSTERS INVOLVED IN THE PRODUCTION OF SECONDARY METABOLITES THROUGH ANALYSIS OF THE GENOME OF *Streptomyces cavourensis* STRAIN CMRP6046

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5.1 ABSTRACT

Antimicrobial resistance (AMR) poses a significant threat to global health, in response, there is an urgent need for novel antimicrobial agents, with microbial natural products (NPs) emerging as promising candidates. *Streptomyces* species are renowned for their ability to produce a diverse array of bioactive compounds, including antibiotics. The biosynthesis of these compounds is orchestrated by biosynthetic gene clusters (BGCs), which encode the machinery for their production, regulation, and transport. Recent efforts have revitalized NP discovery, particularly in unexplored environments like the marine realm, yielding promising strains such as *Streptomyces cavourensis*. This study elucidates the genomic landscape of *S. cavourensis* CMRP6046, aiming to identify genes encoding potential antibacterial agents and other biotechnologically valuable secondary metabolites. Analysis revealed a 7.5 Mb circular genome with 8,016 protein-coding genes, 63 tRNA, and 31 rRNA operons. Comparative genomics with related strains demonstrated a high average nucleotide identity (ANI) value of 96.60%, indicating species delineation. Metabolic pathway analysis highlighted 276 pathway functions, emphasizing metabolism, genetic information processing, and signaling. CMRP6046 exhibited a rich repertoire of 32 putative secondary metabolite biosynthetic gene clusters (BGCs), encompassing RiPPs, PKS, NRPS, terpenes, siderophores, melanin, ectoine, and butyrolactone. Notably, 18 BGCs showed no or low similarities to known clusters, suggesting the strain's potential for novel secondary metabolite production. Moreover, the genome harbors 6 PKS-coding BGCs and 11 RiPPs BGCs, indicating diverse biosynthetic capabilities. This study underscores CMRP6046's significance in natural product discovery and emphasizes the importance of exploring microorganisms in novel niches for biotechnological applications. Future research directions include activating BGCs and employing metabolic profiling for species-level systematics.

Keywords: *Streptomyces*; antimicrobial resistance; whole genome sequencing.

5.2 INTRODUCTION

Antimicrobial resistance (AMR) is a natural phenomenon in which microorganisms such as bacteria, viruses, fungi, and parasites develop the ability to survive the drugs intended to kill them. However, the inappropriate and excessive use of antibiotics in human medicine, livestock farming, and the environment have accelerated the emergence and spread of AMR (Liu et al., 2024). Drug-resistant microbial infections caused 700,000 deaths per year worldwide (Demyanyuk et al., 2022). This poses a significant global threat to human health, and it is of utmost importance to take practical action to address this issue (WHO, 2020).

Currently, it is imperative to discover new compounds with unique mechanisms of action to cure human diseases. One of the primary recommendations for the development of new antibiotics is to encourage drug discovery at an early stage. Therefore, the best alternative to developing a new generation of anti-infective drugs is to discover new microbial natural products (NPs).

These compounds are unmatched in their chemical diversity and effectiveness as antibiotics (Hutchings et al., 2019). This way, bacteria from the phylum Actinomycetota are known for their ability to produce various bioactive secondary metabolites, including antibiotics (Goel et al., 2022), and account for roughly 50% of the antibiotics used in clinical practice (Hutchings et al., 2019). Beyond antibiotics, the genus *Streptomyces* produces a range of other metabolites, including antifungals, antivirals, anticancer agents, antiparasitic agents, immunosuppressants, insecticides, and herbicides (Ngamcharungchit et al., 2023; Yao et al., 2023; Jagannathan et al., 2021).

The secondary metabolites produced by bacteria are low molecular weight compounds that play crucial roles in various biological activities (Zareen et al., 2022) and are synthesized by specific gene clusters called biosynthetic genes (BGCs) (Mehmet et al., 2022). These clusters also contain genes that are responsible for immunity, regulation, and transportation. In bacterial genomes, BGCs are loci that consist of at least two genes required for the biosynthesis of natural products (NPs) (Xia et al., 2022; Rodriguez-Sanchez et al., 2023). BGCs can be classified into three main types based on their distinct structures and biosynthetic pathways. These types include non-ribosomal peptide synthetases (NRPS), polyketide synthase (PKS), and ribosomally synthesized and post-translationally modified peptide (RiPP) (Skinnider e

al., 2020; Crits-Christoph et al., 2021). NRPs and PKSs are vital targets for discovering NPs as they synthesize many antibiotics and drugs (Akomoneh et al., 2023).

Recently, the field of NP discovery has been reinvigorated by the discovery of new antibiotic-producing strains in little-explored environments, including the marine environment, a promising source for finding new drug molecules (Hutchings et al., 2019). *Streptomyces* strains isolated from marine environments produce potent antibacterial compounds. For example, *Streptomyces* sp. EG1 from the Egyptian coast produces mersaquinone (Kim et al., 2020); the *S. xinghaiensis* SCSIO S15077 is isolated that has been found in marine sediment from South China and it produces the bioactive compound tunicamycin (Zhang et al., 2020), and *Streptomyces* sp. EG32 is isolated from the Mediterranean Sea and produces bioactive compounds, such as chlororesistoflavins A and B (Kim et al., 2022).

In this context, this study explores the genome of *Streptomyces cavourensis* CMRP6046 and identify genes with considerable potential for antibacterial efficacy, in addition to other secondary metabolites of biotechnological interest.

5.3 MATERIALS AND METHODS

5.3.1 Bacteria Strain

The strain of *Streptomyces cavourensis* CMRP6046 isolated in (2007) of the intertidal zone of Ilha do Mel, in the coastal region of the state of Paraná, Brazil (25°20′S, 48°20′W and 25°35′S, 48°35′W) previously identified and characterized (Porsani et al., 2017). The bacterial strain was deposited in the Microbiological Collections Centre of the Taxonline Network of Paraná (CMRP/Taxonline) (https://www.cmrp-taxonline.com/catalogue). The strain was stored in a -80ºC degree on Glucose Yeast Extract liquid medium (GY) with 30% glycerol.

5.3.2 DNA Extraction

The strain of *S. cavourensis* CMRP6046 was grown on Glucose Yeast Extract Agar (GY) 28ºC for 7 days. DNA extraction was performed the mixture of silica and celite cetyltrimethylammonium bromide (CTAB) 2:1 method with chloroform: isoamyl

alcohol (CIA) 24:1 v/v (Moreira et al., 2010). The DNA concentration was quantified by Nanodrop (2000) and Qubit Fluorometer (Invitrogen).

5.3.3 Library construction

Library construction used Rapid Barcoding Kit 24 V14 (MinION Nanopore platform) adapted with a step to select long fragments using Long Fragment Buffer and sequencing was performed by MinION Mk1C (Nanopore) with a FlowCell R.10.

5.3.4 Nanopore Sequencing, Assembling and Annotation

Nanopore sequencing data were acquired in FASTQ format with long reads. The quality of the nanopore raw data was assessed using NanoPlot v1.32.0 (De Coster et al., 2018) for visualization and basic data statistics evaluation. Subsequently, the long reads raw data were pre-processed to remove adapters, low-quality regions, and sequences shorter than 1000 bases using Porechop v0.2.4 (Wick, 2017). Then, the pre-processed reads were corrected using Medaka v1.0.3 (https://github.com/nanoporetech/medaka) with default parameters. Next, the corrected reads were conducted using Flye assembler v2.9.3 (Kolmogorov et al., 2020) with parameters optimized for hybrid data. The resulting contigs were polished using Racon v1.4.3 (Vaser et al., 2017) with the corrected reads as input. Following assembly, the quality of the assembly was evaluated using standard metrics, including N50, number of contigs, total assembly length, and percentage of reads mapped back to the assembly. Additionally, the quality of the assembly was visually verified using Bandage v0.8.1 (Wick et al., 2015) to inspect the assembly graph structure.

The genome of strain CMRP6046 was used to determine the taxonomic parameters between its closely related strains. This process involved average nucleotide identity based FastANI (Jain et al., 2018) using a database with 500 species of the genus *Streptomyces.* In addition, the calculation of the MUMmer Average Nucleotide Identity (ANIm) was performed using the web-based analysis platform, available at: <https://jspecies.ribohost.com/jspeciesws/>. We used the standard parameters and the platform pipeline that allows evaluating the genetic similarity between the genome of the species of interest against the genome of the reference species. For ANIm, genomic sequences are homologated through Maximal Unique

Matches (MUMmer), which identify regions of unique sequence in each genome, as well as regions of similarity between them. ANIm was calculated from the MUMmer alignment using the formula: ANIm = (number of identical nucleotide pairs in homologous regions / total length of homologous regions) x 100. The algorithms above are presented as a cutoff point for determining new species with a threshold value > 95~96%. Therefore, the investigated genome belongs to the same species when >96%, while the value below indicates a new species compared to all the most related types (Lee et al., 2016, Yoon et al., 2017).

The metabolic pathways was functionally annotated and identified using eggNOG v5.0 (Huerta-Cepas et al., 2019), a database of orthology relationships, functional annotations, and gene evolutionary histories, as well as the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Aramaki et al., 2019; Kanehisa and Sato., 2020), a database resource used to gain an in-depth understanding of the high-level functions and utilities of the biological system.

The resulting contigs were then functionally annotated using the server Rapid Annotations using Subsystems Technology - RAST (Aziz et al., 2008). Furthermore, the identification of biosynthetic gene clusters was performed using AntiSMASH v7.1.0 bacterial version with detection strictness set to “relaxed” and extra features selected to “ActiveSiteFinder, KnownClusterBlast, SubClusterBlast” (Blin et al., 2023). Initially, the annotated gene sets were extracted from the functional annotation results. The protein sequences corresponding to the annotated genes were then extracted and organized with Augustus v.3.4.0 (Stanke et al., 2008). Subsequently, the protein sequences were submitted to OrthoFinder (Emms and Kelly, 2019) for analysis. OrthoFinder conducted a search for orthologous groups using a combination of sequence similarity methods and phylogenetic trees. The orthologous groups identified by OrthoFinder were reviewed to identify specific genes of interest. The identified orthologous genes were grouped into gene families based on their sequence and functional similarity.

5.3.5 Data availability

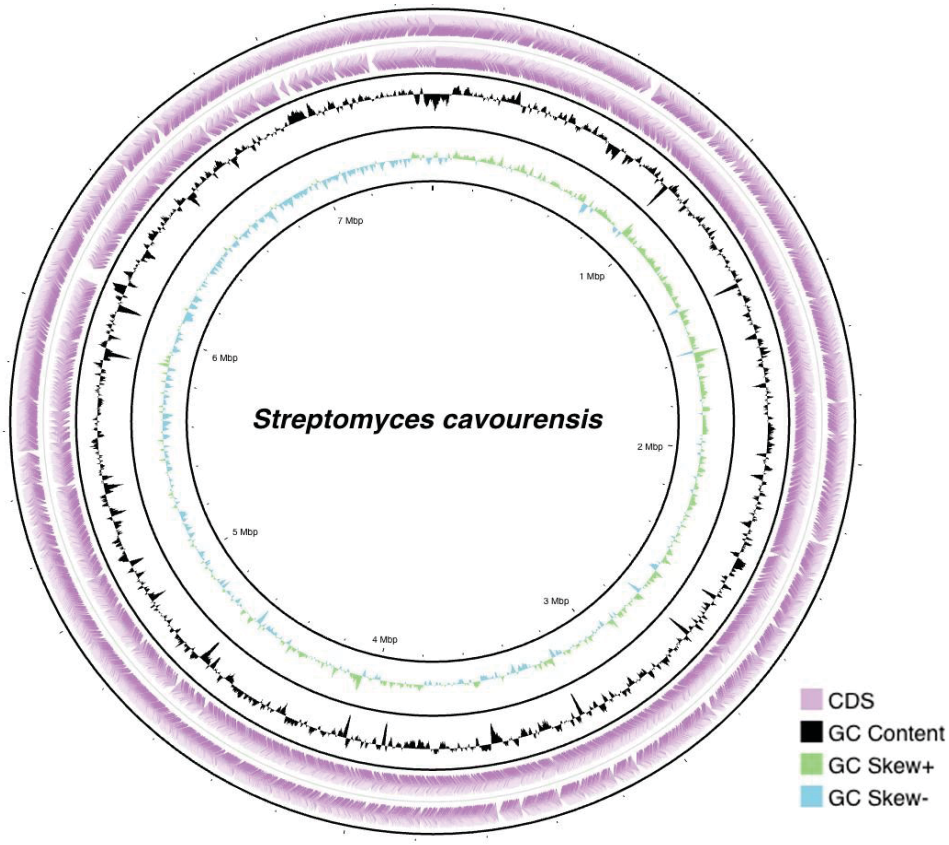
The *S. cavourensis* CMRP6046 genome has been deposited in NCBI GenBank under accession numbers CP152096-CP152101.

5.4 RESULTS

5.4.1 Assembly and Annotation of genome *Streptomyces cavourensis* CMRP6046

A total of 35,368 raw reads were generated by Nanopore. The final assembly yielding a 7,504,578 Mb circular genome with an average GC content of 71.9%, and plasmid (8,535 - 175,780 - 69,6) (Figure 8).

FIGURE 8 - THE CIRCULAR GENOME *Streptomyces cavourensis* CMRP6046



The figure was created using Proksee (Grant et al., 2023).

Notes: The circular diagram of the genome of *Streptomyces cavourensis* CMRP6046 illustrates the location of the CDS (protein-coding genes), the GC content, and the GC slope throughout the genome. The layers, beginning with the outermost, represent these features.

The chromosome contained 12 contigs encoding 8,016 protein-coding genes (CDSs), there are 63 tRNA and 31 rRNA operons (Table 9). The genome assembly was validated using BUSCO, leading to 135 complete single-copy (91.22%), seven duplicated (4.73%), five missing (3.38%), and one fragmented (0.68) BUSCOs.

TABLE 9 - *Streptomyces cavourensis* CMRP6046 GENOME DATA ASSEMBLY AND QUALITY

	Genome	**Plasmid**
Genome size (bp)	7,504,578	8,535 - 175,780
GC content (%)	71.9	69,6
Coverage (%)	97	-
Coding protein genes	8,016	-
tRNA genes	63	-
rRNA operons	31	-
Genes (total)	8,110	-

SOURCE: The author (2024).

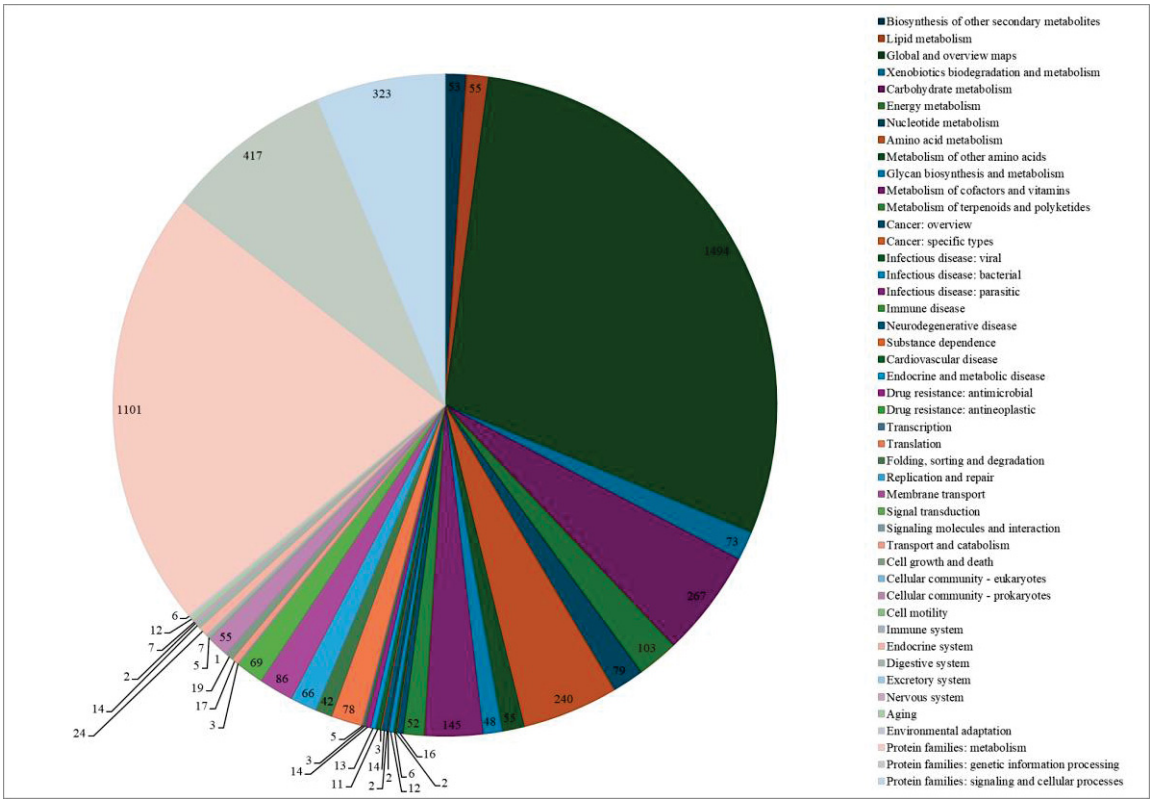
5.4.2 Taxonomic status

The fastANI was used to analyze the average nucleotide identity (ANI) of *Streptomyces cavourensis* CMRP6046 and its closely related strains. The result revealed that the ANI value of S. *cavourensis* CMRP6046 and *Streptomyces cavourensis* 1AS2a was 96.60%. As the ANI value was higher than 95%, which is the level used to distinguish between bacterial species (Jain et al., 2018), the strain can be considered *Streptomyces cavourensis* species among the currently known strains with whole genome sequencing available. As well as the 96.15% value of MUMmer (ANIm) which indicated that the strain CMRP6046 is *Streptomyces cavourensis* when compared to the reference strain (*Streptomyces cavourensis* - GCA_002804165.1_ASM280416v1_genomic.fna).

5.4.3 Metabolic pathways and functions of genes annotations of the *Streptomyces. cavourensis* CMRP6046

Functional gene analysis using KEGG categories revealed that the number of genes involved in metabolic processes was higher than the number of function-related genes annotated in KEGG (1,556), which implies that the producer possesses powerful metabolic capacities. All the genes are mainly concerned around 276 pathway functions of which the maximum number of genes is involved in protein families: metabolism (1,101), genetic information processing (417) and signaling and cellular processes (323). This is followed majorly by carbohydrate metabolism (232) and 276 amino acid metabolism (260) (Figure 9).

FIGURE 9 - OVERWIEW OF METABOLIC PATHWAYS AND FUNCTIONS OF GENES ANNOTATIONS OF STRAIN *Streptomyces cavourensis* CMRP6046



SOURCE: The author (2024).

In addition, there are 52 genes associated with the pathways of metabolism of terpenoids and polyketides responsible for antibiotic synthesis, ansamycins (01051), enediyne (01059), tetracycline (00253), along with other potential antibiotics. In addition, 52 genes belong to the pathways of biosynthesis of secondary metabolites: monobactam biosynthesis (00261); streptomycin biosynthesis (00521); penicillin and cephalosporin biosynthesis (00311); Carbapenem biosynthesis (00332); novobiocin biosynthesis (00401); Prodigiosin biosynthesis (00333) and biosynthesis of various antibiotics: Kanosamine, Aurachin, Bacilysin, Puromycin, Dapdiamides, Fosfomycin, Cremeomycin, Fumagillin, Pentalenolactone, Terpentecin, Roseoflavin, Cycloserine (KO00998) (S.M. 6).

5.4.4 Biosynthetic gene clusters (BCGs) for secondary metabolites of the *Streptomyces cavourensis* CMRP6046

AntiSMASH analysis resulted in the identification of 32 BCGS regions encoding secondary metabolites of the *S. cavourensis* CMRP6046. Meanwhile, 12

suggested gene clusters showed high similarity (> 80%) with reported gene clusters: alkylresorcinol, naringenin, ectoin, desferrioxamine B, keywimysin, bafilomycin B1, SGR_PTM, griseobactin, geosmin, isorenieratene, coelicelin and AmfS. Among the remaining 20 BGCs, 2 exhibited some level of similarities (50%-70%) with known BGCs such as hopene and streptamidine.

In addition, the results of the antiSMASH prediction showed that 18 of the 32 predicted BGCs were less than 50% similar to known BGCs, indicating that strain CMRP6046 contains many undescribed BGCs responsible for new compounds: bottromycin A2, lactazole, aborycin, steffimycin D, azotobactin D, kinamycin, coelimycin P1, diazaquinomycin A/diazaquinomycin E/diazaquinomycin F/diazaquinomycin G, SCO-2138, enteromycin, JBIR-126 and istamycin and 4 unidentified BGCs. Thus, the BGC encoding a T3PKS and a BCG encoding a RiPP were predicted on the plasmid. Details of the most similar gene clusters, similarity percentages and largest gene clusters are provided in (S.M. 5).

5.5 DISCUSSION

The Gram-positive, aerobic, spore-forming and filamentous actinobacterial strain *Streptomyces cavourensis* CMRP6046 was isolated marine sediment the intertidal zone of Ilha do Mel, in the coastal region of the state of Paraná, Brazil.

The genome of *Streptomyces* spp. typically comprises a single linear chromosome with an average size of approximately 8 Mb, although sizes ranging from 6.8 Mb to 11.9 Mb have been documented. Notably, their DNA exhibits a high guanine and cytosine (G+C) content, averaging around 70%, but varying between 69% and 78%. This distinguishes them from bacteria like *Staphylococcus* and *Bacillus*, which typically have a G+C content of around 50% (Williams et al., 1983; Hopwood, 2006; Chandra and Wang, 2013; Chater, 2016).

In 1976, genetic research into bioactive compound production in *Streptomyces* spp. began with the discovery of plasmid pSV1, which enabled the biosynthesis of methylenomycin A and conferred resistance to the antibiotic in *Streptomyces coelicolor* A3. This breakthrough was achieved through conventional plasmid transfer techniques via conjugation in mixed cultures (Wright and Hopwood, 1976).

In the early 2000s, the complete genome of *S. coelicolor* A3 was sequenced from cosmid libraries, marking the first genome obtained for the genus. Moreover, it

facilitated the discovery of new drugs through genetic engineering (Bentley et al., 2002). Subsequent studies have further analyzed the genome of this microorganism (Faddetta et al., 2022; Dulermo et al., 2023; Kronheim et al., 2023).

The whole genome mining of *S. cavourensis* strain CMRP6046, following research already reported in the literature on several isolates of the same genus clearly shows that the annotations are within average values. Through bioinformatic tools, the CMRP6046 strain has presented a total of 35,368 raw reads generated by Nanopore. The final assembly yielded a 7.5 Mb circular genome with an average GC content of 71.9%. The chromosome contained 12 contigs encoding 8,016 protein-coding genes (CDSs), there are 63 tRNA and 31 rRNA operons (Table 9). In addition, to determine whether CMRP6046 strain does indeed represent a genomic species, the fastANI value and MUMmer (ANIm) analysis was employed to further clarify the relatedness between CMRP6046 and *S. cavourensis* 1AS2a isolated to rhizosphere of the *Triticum aestivum*, Brazillian Cerrado Forest soil. The fastANI value and ANIm were 96.60% and 96.15%, respectively, which are more than the threshold of 95–96% ANI cut-off widely accepted for delineating prokaryotic species (Zhao et al., 2021).

The identification of compounds from the genome is essential for guiding in vitro experiments. By selecting microorganisms with the greatest potential for producing new bioactive compounds, we can make these experiments more effective (Quach et al., 2023, Klykleung et al., 2023). Another crucial aspect is understanding the genomic profile of *Streptomyces* (Jia et al., 2023), this is essential because different regulators control the secondary metabolite production pathways (Yan et al., 2023). Although secondary metabolite expression is low or silent, new tools allied to the genome can be applied (Psenicnik et al., 2024). Furthermore, biodiversity and metabolic profile studies are crucial for identifying new species that can be bioprospecting in the future (Liu et al., 2023).

Streptomyces species are thought to acquire biosynthetic gene clusters through horizontal gene transfer, thereby enabling them to quickly start producing various secondary metabolites (Chater et al., 2010). The intertidal regions where *Streptomyces cavourensis* CMRP6046 was isolated have extreme environmental conditions that favor the development of microorganisms that produce complex secondary metabolites, such as actinobacteria. The intertidal region is characterized by high salinity, humidity, UV radiation, temperature, mechanical action of the tides, and nutrient deficiency (Porsani et al., 2017).

The KEGG pathway database was useful for systematic analyses of the metabolic pathways and functions of gene products. Such analyses provide information regarding molecular interaction networks and the unique changes to individual biological pathways in organisms. All the genes were mainly concerned around 276 pathway functions of which the maximum number of genes is involved in protein families: metabolism (1,101), genetic information processing (417) and signaling and cellular processes (323). This is followed majorly by carbohydrate metabolism (267) and amino acid metabolism (240) (Figure 9). There are 52 genes associated with the pathways of metabolism of terpenoids, and polyketides and 53 genes associated with the biosynthesis of other secondary metabolites (S.M. 6 and Figure 9).

The *in-silico* observations indicate that *S. cavourensis* CMRP6046 exhibits a greater number of metabolic pathways, and a greater number of genes involved in pathways related to the production of secondary metabolites. The endophyte *S. cavourensis* strain YBQ59 was isolated from the root of the medicinal plant *Cinnamomum cassia* Presl in Vietnam, analysis of the genome annotation based KEGG database revealed at least 140 distinct metabolic pathways. Notably, 717 enzymes linked to secondary metabolic biosynthesis were predicted, among which 158 enzymes are directly involved in antibiotic biosynthetic pathways (Nguyen et al., 2018). A new actinobacteria *S. cavourensis* TJ430 was isolated from the mountain soil collected from the southwest of China. There are 237 proteins predicted for the process of secondary metabolites biosynthesis, transport, and catabolism, reflecting more complex “secondary metabolic” pathways (Wang et al., 2018).

To evaluate the secondary metabolite biosynthetic potential of the genome of strain *S. cavourensis* CMRP6046 was analyzed with antiSMASH. A total of 32 putative secondary metabolites BGCs were detected (S.M. 5). The *S. cavourensis* CMRP6046 comprises 15 different types of BGCs, including those coding for ribosomally synthesized and post-translationally modified peptides (RiPPs) (thiopeptide, lanthipeptide, lasso peptide, RRE-containing, and RiPP-like), PKS, NRPS, hydrogen-cyanide, bottromycin, terpene, siderophore, melanin, ectoine, and butyrolactone. This highlights the potential of CMRP6046 to produce novel secondary metabolites.

The *S. cavourensis* CMRP6046 isolated from marine sediment had a higher number of BCGS in comparison to reports in the literature, the *S. cavourensis* strain 2BA6PGT that also was isolated from sediment from the bottom of the salt lake

Verkhnee Beloe (Buryatia, Russia) complete genome has total of 27 BGCs were identified, 13 of which matched known clusters with 70–100% similarity of isorenieratene, alkylresorcinol, melanin, valinomycin/montanastatin, SGR PTMs, bafilomycin B1, SAL-2242, keywimysin, desferrioxamine B, ectoine, coelichelin, streptobactin, and geosmin (Chong et al., 2023). To identify the BGCs of *S. cavourensis* 1AS2a isolated from wheat rhizosphere in the Brazilian Neotropical savanna, which identified 30 BGCs, 10 of which matched known clusters for ectoine, desferrioxamine B, SRO 15-2005, Amfs, macrotetrolide, bafilomycin, SGR_PTMs, melanin, alkylresorcinol, and isorenieratene; these had 100% similarity and two clusters encoding griseobactin and coelichelin at >70% (Hoyos et al., 2019).

A remarkable feature of the strain CMRP6046 genome is the presence of 6 PKS-coding BGCs, encoding three different kinds of PKS (three PKS I and three PKS III). The three PKS I cluster (26, 27 and 29) showed 100% similarity with BGCs encoding bafilomycin B1, SGR_PTM and griseobactin. The type III PKS genes clusters 4, 6 and 32 showed, respectively, 100%, 100% and 4% similarities with the alkylresorcinol, naringenin and istamycin. The istamycin is a member of the aminoglycoside compounds that predominantly display antimicrobial activity all mediated by inhibiting translation on the 30S subunit of the bacterial ribosome (Piepersberg et al., 2007).

Eleven BGCs are involved in the biosynthesis of RiPPs (thiopeptide, lanthipeptide, lasso peptide, RRE-containing, and RiPP-like). Two clusters (18 and 19) of eleven RiPPs BGCs showed a high similarity lassopeptide of keywimysin (100%) and to class III lanthipeptide of AmfS (80%), The AmfS comprises biological surfactants that positively regulate the formation of aerial mycelia (Ueda et al., 2002). In the remaining nine RiPP BGCs, six BGCs (1, 2, 5, 9, 12 and 22) showed low similarities (<60%) to the known BGCs, and three BGCs (10, 13 and 23) did not match with known gene clusters. These findings revealed that strain CMRP6046 has the potential to produce the novel RiPPs.

The clusters in *S. cavourensis* CMRP6046, 3, 7, 25 and 30, are terpene BGCs, assumed to be like the BGCs of isorenieratene, steffimycin D, hopene and geosmin, respectively. Except for cluster 3 and 30, which shows BGC similarity with isorenieratene (85%) and geosmin (100%), the other three clusters, 7 (19%), 25 (69%), and 30 (30%), showed low similarities with known BGCs, and cluster 20 did not match

with known gene clusters, indicating that the strain also has the potential to produce novel terpene compounds.

The remaining clusters *S. cavourensis* CMRP6046, 8, 11, 14, 15, 16, 17, 21, 24 and 28, except for cluster 8 which shows BCG similarity with ectoine (100%) - a secondary metabolite that confers protection for the bacterial cell in extreme temperature conditions (Goel et al., 2022) - the other clusters are NRPS BCGs. The Clusters 11 (100%) and 28 (81%) show similarity with desferrioxamine B and celichelin, respectively. Other clusters, 14 (8%), 15 (16%), 16 (47%), 17 (15%), 21 (16%), 24 (7%) showed low similarity with the known BGCs enteromycin, azotobactin D, showdomycin, diazaquinomycin, kinamycin and JBIR-126, respectively, indicating that the strain also has the potential to produce new compounds.

A positive correlation has been identified between the isolation and discovery of new bioactive compounds in actimomycetes, particularly in the genus *Streptomyces.* The present study was designed to establish the taxonomic status of this new isolate and, in addition, to describe its biosynthesis potential for producing new natural products by means of genome mining. The results of *in silico* analysis, as well as the ANI and MUMmer values, led to the conclusion that strain CMRP6046 represents the specie *Streptomyces cavourensis*.

A high percentage of this genome is dedicated to secondary metabolite production, as indicated by the length of the BGC-related sequences. Thirty-two secondary metabolite BGCs in strain CMRP6046 were distributed across 15 different types. Four of them show no similarity to any reference BGCs and twelve of them show moderate and low similarity BCGs, while twelve BGCs representing 62.5% of related genes showed high similarity to homologs from known BGCs. This indicates that strain CMRP6046 has the potential to produce new secondary metabolites.

In conclusion, the exploration of microorganisms in new niches is a field of study that aims to identify novel genes and natural products. This *Streptomyces cavourensis* shows great potential to produce novel natural products that could be used by the medical and agricultural industries. Since, in recent years, several approaches for activating the BGCs have been developed (Liu et al., 2020; Nguyen et al., 2020), future studies should focus on not only the discovery of uncultured or new microorganisms but also how to isolate more bioactive products with new methods and to use the metabolic profiling in species-level systematics research.

CONFLICT OF INTERESTS

The authors declare no competing interest.

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SUPPLEMENTARY MATERIAL 5 - OVERVIEW OF 32 SECONDARY METABOLIC BIOSYNTHESIS GENE CLUSTERS OF *Streptomyces cavourensis* CMRP6046 STRAIN DETECTED BY antiSMASH 7.1

Cluster	Type	From	To	Most similar known cluster	Similarity	%	MIBiG accession	Reference strain	Biological activity
1	bottromycin	76,716	97,51	bottromycin A2	RiPP:Bottromycin	36	BGC0000469	*Streptomyces* sp. BC16019	Antibiotic
2	NRPS, LAP, thiopeptide	58,146	104,076	lactazole	RiPP:Thiopeptide	33	BGC0000606	*Streptomyces lactacystinaeus*	Antibiotic
3	terpene	114,647	140,228	isorenieratene	Terpene	85	BGC0000664	S. *griseus subsp. griseus* NBRC 13350	Antioxidant
4	T3PKS	213,264	254,316	alkylresorcinol	Polyketide	100	BGC0000282	S. *griseus subsp. griseus* NBRC 13350	Prevention of tumor
5	melanin,RiPP-like	287,929	310,582	streptamidine	RiPP:Other	58	BGC0002115	*Streptomyces albidoflavus* J1074	Unknown
6	T3PKS	423,309	464,427	naringenin	Polyketide:Type III polyketide	100	BGC0001310	*Streptomyces clavuligerus* ATCC 27064	Antioxidant
7	terpene	991,273	1,012,367	steffimycin D	Polyketide:Type II polyketide+Saccharide:Hybrid/tailoring saccharide	19	BGC0000273	*Streptomyces steffisburgensis*	Antitumor
8	ectoine	1,434,417	1,444,815	ectoine	Other	100	BGC0000853	*Streptomyces anulatus*	Natural cell protectant
9	RRE-containing	2,043,465	2,063,791	SCO-2138	RiPP	14	BGC0000595	*Streptomyces coelicolor* A3(2)	–
10	lanthipeptide-class-ii, lanthipeptide-class-iii	2,382,104	2,413,252	–	–	–	–	–	–
11	NI-siderophore	2,458,295	2,488,072	desferrioxamin B	Other	100	BGC0000941	S. *griseus subsp. griseus* NBRC 13350	Antitumor
12	NRPS-like	2,552,568	2,596,980	bottromycin A2	RiPP:Bottromycin	39	BGC0000469	*Streptomyces* sp. BC16019	Antibiotic
13	thiopeptide, LAP, RRE-containing	2,826,525	2,861,405	–	–	–	–	–	–

Cluster	Type	From	To	Most similar known cluster	Similarity	%	MIBiG accession	Reference strain	Biological activity
14	ectoine	2,865,051	2,875,452	enteromycin	Polyketide+NRP	8	BGC0002499	S. *achromogenes subsp. streptozoticus*	Antibacterial
15	NRPS	3,098,927	3,153,204	azotobactin D	NRP	16	BGC0002433	*Azotobacter vinelandii* DJ	Iron chelator
16	Ectoine butyrolactone	3,460,806	3,476,041	showdomycin	Other	47	BGC0001778	*Streptomyces showdoensis*	Antibiotic, antitumor
17	NRPS-like	3,543,539	3,585,389	diazaquinomycin A diazaquinomycin E diazaquinomycin F diazaquinomycin G	Other:Shikimate-derived	15	BGC0001850	*Streptomyces* sp. F001	Cytotoxicity against some solid tumors
18	lassopeptide	4,212,913	4,235,517	keywimysin	RiPP	100	BGC0001634	*Streptomyces* sp. NRRL F-5702	Unknown
19	lanthipeptide-class-iii	5,108,610	5,131,360	AmfS	RiPP: Lanthipeptide	80	BGC0000496	S. *griseus subsp. griseus* NBRC 13350	Onset of aerial-mycelium formation, antibiotic production
20	terpene	5,521,677	5,542,654	–	–	–	–	–	–
21	NI-siderophore	5,944,168	5,976,657	kinamycin	Polyketide	16	BGC0000236	*Streptomyces murayamaensis*	Antibiotic
22	hydrogen-cyanide	6,156,608	6,169,881	aborycin	RiPP	21	BGC0002285	*Streptomyces* sp. ZS0098	Antibiotic
23	RiPP-like	6,297,213	6,308,520	–	–	–	–	–	–
24	NRPS-like,NRPS	6,440,664	6,488,799	JBIR-126	NRP	7	BGC0001368	*Streptomyces* sp. NRRL F-4474	Unknown
25	terpene	6,900,348	6,927,455	hopene	Terpene	69	BGC0000663	*Streptomyces coelicolor* A3(2)	Unknown
26	T1PKS	6,958,730	7,053,714	bafilomycin B1	Polyketide: Modular type I polyketide	100	BGC0000028	*Streptomyces lohii*	Antibacterial, antifungi, antinsect, antinematode antiprotozoan
27	T1PKS, NRPS-like,RiPP-like	7,074,311	7,123,702	SGR_PTM	Other+Polyketide	100	BGC0002365	*Streptomyces* sp. SCSIO 40010	Cytotoxic

Cluster	Type	From	To	Most similar known cluster	Similarity	%	MIBiG accession	Reference strain	Biological activity
28	NRP-metallophore, NRPS,melanin	7,153,217	7,211,753	coelichelin	NRP	81	BGC0000325	*Streptomyces coelicolor* A3(2)	Iron chelator
29	NRP-metallophore, NRPS, transAT-PKS, T1PKS, PKS-like	7,219,410	7,335,373	griseobactin	NRP	100	BGC0000368	*Streptomyces* sp. ATCC 700974	Iron chelator
30	terpene	7,338,484	7,360,697	geosmin	Terpene	100	BGC0001181	*Streptomyces coelicolor* A3(2)	Earthy smelling substance
31	butyrolactone	7,386,829	7,397,698	coelimycin P1	Polyketide: Modular type I polyketide	16	BGC0000038	*Streptomyces coelicolor* A3(2)	Yellow pigment

SOURCE: The author (2024).

Legend: BGCs - biosynthetic gene clusters; %- indicates the proportion of genes with similarities; T1PKS – type I polyketide synthase; T2PKS – type II polyketide synthase; T3PKS – type III polyketide synthase; NRPS – non-ribosomal peptide synthase; RiPP – ribosomally synthesized and post-translationally modified peptide; - not determined.

SUPPLEMENTARY MATERIAL 6 -METABOLIC PATHWAYS AND FUNCTIONS OF GENES ANNOTATIONS OF THE *Streptomyces cavourensis* CMRP6046 DETECTED BY THE KYOTO ENCYCLOPEDIA OF GENES AND GENOMES (KEGG) DATABASE

Map	Pathway	Module	KEGG ID	Name	Remark
902	Geraniol biosynthesis		C01500	Geraniol	Monoterpenoid
	Myrcene biosynthesis		C06074	Myrcene	
	Limonene biosynthesis		C00521	(S)-Limonene	
			C06099	(R)-Limonene	
	Menthol biosynthesis		C00400	Menthol	
	Carvone biosynthesis		C01767	(-)-Carvone	
			C11383	(+)-Carvone	
	Pinene biosynthesis		C06308	alpha-Pinene	
			C06307	beta-Pinene	
	Camphene biosynthesis		C06305	(-)-Camphene	
			C06304	(+)-Camphene	
	Camphor biosynthesis		C01765	(+)-Borneol	
			C00808	(+)-Camphor	
	Secologanin biosynthesis		C01433	Loganin	Iridoid/secoiridoid
			C01852	Secologanin	
909	Farnesol biosynthesis		C01126	(2E,6E) -Farnesol	Sesquiterpenoid
			C03220	(2Z,6E) -Farnesol	
	Germacrene biosynthesis		C16141	Germacrene A	
			C09672	Germacrene B	
			C19747	Germacrene C	
			C16142	Germacrene D	
	Bisabolene biosynthesis		C19749	alpha-Bisabolene	
			C16775	beta-Bisabolene	
			C16814	(Z)-gamma-Bisabolene	
			C19748	(E)-gamma-Bisabolene	
	Humulene biosynthesis		C09684	alpha-Humulene	
			C16829	gamma-Humulene	
	Cadinene biosynthesis		C06394	delta-Cadinene	
	Hopane biosynthesis		C06309	Hopan-22-ol	Triterpenoid
			C06310	Hop-22(29) -ene	
904	Gibberellin biosynthesis	M00927	C00859	Gibberellin A1	Plant hormone
		M00928	C01699	Gibberellin A3	
		M00929	C11864	Gibberellin A4	
	Labdane biosynthesis		C09183	Sclareol	Diterpenoid
			C18224	Stemod-13(17) -ene	
	Abietate biosynthesis		C06087	Abietate	
	Paclitaxel biosynthesis		C11900	Baccatin III	Taxol, diterpenoid
			C07394	Paclitaxel	
906	alpha-Carotene biosynthesis		C05433	alpha-Carotene	Provitamin A carotenoid
	beta-Carotene biosynthesis	M00097	C02094	beta-Carotene	
	Spirilloxanthin biosynthesis		C15881	Spirilloxanthin	Carotenoid
			C15888	Tetrahydrospirilloxanthin	
	Spheroidene biosynthesis		C15900	Spheroidene	

			C15903	Spheroidenone	
	Okenone biosynthesis		C16280	Okenone	
	Bacterioruberin biosynthesis		C22349	Bacterioruberin	
	Astaxanthin biosynthesis		C08580	Astaxanthin	
	Myxoxanthophyll biosynthesis		C15937	(3R,2'S)-Myxol 2'-(2,4-di-O-methyl-alpha-L-fucoside)	Carotenoid glycoside
	Xanthophyll cycle		C06098	Zeaxanthin	Xanthophyll
			C08579	Antheraxanthin	
			C08614	Violaxanthin	
	Lutein biosynthesis		C08601	Lutein	
	Neurosporaxanthin bisynthesis		C08607	Neurosporaxanthin	
	Diapocarotene biosynthesis		C16148	Staphyloxanthin	
	Abscisic acid biosynthesis	M00372	C06082	Abscisic acid	Plant hormone
905	Brassinosteroid biosynthesis	M00371	C15794	Castasterone	Plant hormone
			C08814	Brassinolide	
981	Juvenile hormone biosynthesis		C09694	Juvenile hormone III	Insect hormone
	Ecdysone biosynthesis		C00477	Ecdysone	
908	Zeatin biosynthesis		C00371	trans-Zeatin	Plant hormone
			C15545	cis-Zeatin	
522	Tylosin biosynthesis	M00773	C01457	Tylosin	Macrolide antibiotic
	Mycinamicin biosynthesis	M00934	C15680	Mycinamicin II	
	Erythromycin biosynthesis	M00774	C01912	Erythromycin A	
			C06653	Erythromycin B	
	Oleandomycin biosynthesis	M00775	C01946	Oleandomycin	
	Pikromycin/methymycin biosynthesis	M00776	C11999	Pikromycin	
	Avermectin biosynthesis	M00777	C11984	Avermectin A1a	Macrolide pesticide
1051	Rifamycin biosynthesis		C12044	Rifamycin SV	Ansamycin antibiotic
	Ansamitocin biosynthesis		C12045	Ansamitocin P-3	Ansamycin antitumor antibiotic
1059	9-Membered enediyne biosynthesis	M00824	C11438	C-1027	Enediyne antitumor antibiotic
			C12049	Neocarzinostatin	
			C21301	Kedarcidin	
	10-Membered enediyne biosynthesis	M00825 M00833	C11469	Calicheamicin gamma(1)I	
1057 253	Benzoisochromanequinone biosynthesis	M00779	C06691	Actinorhodin	Aromatic polyketide
			C12437	Medermycin	
			C06799	Granaticin	
	Tetracycline biosynthesis	M00778	C06570	Tetracycline	
		M00780	C06571	Chlortetracycline	
		M00823	C06624	Oxytetracycline	
	Angucycline biosynthesis		C18685	Landomycin E	
			C18822	Landomycin A	
			C12413	Urdamycin A	
			C18680	Jadomycin A	
			C12395	Jadomycin B	
	Anthracycline biosynthesis	M00781	C01907	Daunorubicin	
			C01661	Doxorubicin	

			C18638	Aclacinomycin A	
			C18642	Rhodomycin B	
			C18633	Nogalamycin	
	Mithramycin biosynthesis	M00782	C12389	Mithramycin	
1057 253	Tetracenomycin biosynthesis	M00783 M00784	C06801	Tetracenomycin C	
			C12380	Tetracenomycin X	
			C12381	Elloramycin A	
1053	Enterobactin biosynthesis		C05821	Enterobactin	Siderophore
	Vibriobactin biosynthesis		C06769	Vibriobactin	
	Yersiniabactin biosynthesis		C12038	Yersiniabactin	
	Petrobactin biosynthesis		C22273	Petrobactin	
1055	Vancomycin biosynthesis		C06689	Vancomycin	Glycopeptide antibiotic
	Chloroeremomycin biosynthesis		C12014	Chloroeremomycin	
940	Monolignol biosynthesis	M00039	C02646	4-Coumaryl alcohol	Monolignol
			C00590	Coniferyl alcohol	
			C02325	Sinapyl alcohol	
940 941	Flavanone biosynthesis	M00940 M00137	C09827	Pinocembrin	Flavanone
			C09762	Liquiritigenin	
			C09616	Butrin	
			C00509	Naringenin	
			C05631	Eriodictyol	
			C05911	Pentahydroxyflavanone	
941 944	Flavone biosynthesis		C01477	Apigenin	Flavone
			C01514	Luteolin	
	Flavonol biosynthesis		C05903	Kaempferol	Flavonol
			C00389	Quercetin	
			C11620	Syringetin	
941 942	Anthocyanin biosynthesis	M00138	C05904	Pelargonidin	Anthocyanidin/anthocyanin
			C05905	Cyanidin	
			C05908	Delphinidin	
943	Isoflavonoid biosynthesis	M00941	C10208	Daidzein	Isoflavonoid
			C06563	Genistein	
	Pterocarpan biosynthesis	M00942	C10503	Medicarpin	Pterocarpan
945	Stilbenoid biosynthesis		C03582	Resveratrol	Stilbenoid
			C10287	Pterostilbene	
			C01745	Pinosylvin	
	Diarylheptanoid biosynthesis		C10443	Curcumin	Diarylheptanoid
	Gingerol biosynthesis		C10462	6-Gingerol	Gingerol, pungent
901	Psilocybin biosynthesis	M00962	C07576	Psilocybin	Indole alkaloid
			C08312	Psilocin	
	Ergot alkaloid biosynthesis	M00963 M00964	C07544	Ergotamine	
			C20436	Fumigaclavine A	
			C20437	Fumigaclavine B	
			C20438	Fumigaclavine C	
	Monoterpene indole alkaloid biosynthesis		C06542	Ajmaline	
			C02074	Raucaffricine	
			C09239	Sarpagine	
		M00965	C01626	Vindoline	
			C07201	Vinblastine	
			C07204	Vincristine	
403		M00661	C20530	Paspaline	Indole diterpene alkaloid

	Indole diterpene alkaloid biosynthesis		C20555	Aflatrem	
			C20550	Lolitrem E	
			C20551	Lolitrem B	
950	Isoquinoline alkaloid biosynthesis	M00943	C02105	Reticuline	Isoquinoline alkaloid
		M00944	C01516	Morphine	
		M00945	C06162	Sanguinarine	
		M00946	C09592	Noscapine	
960	Tropane alkaloid biosynthesis		C01851	Scopolamine	Tropane alkaloid
			C01416	Cocaine	
	Piperidine alkaloid biosynthesis		C03882	Piperine	Piperidine alkaloid
			C10171	(-) -Sedamine	
			C07475	Lobeline	
	Pyridine alkaloid biosynthesis		C06524	Nornicotine	Pyridine alkaloid
960			C01004	Trigonelline	
			C10126	Anatabine	
	Quinolizidine alkaloid biosynthesis		C10773	Lupinine	Quinolizidine alkaloid
	Indolizidine alkaloid biosynthesis		C06185	Slaframine	Indolizidine alkaloid
			C10173	Swainsonine	
	Pyrrolizidine alkaloid biosynthesis		C15612	Senecionine N-oxide	Pyrrolizidine alkaloid
	Pyrrolidine alkaloid biosynthesis		C01679	Hygrine	Pyrrolidine alkaloid
			C06521	Cuscohygrine	
965	Betalain biosynthesis	M00961	C08540	Betanin	Betacyanin
			C08557	Miraxanthin-V	Betaxanthin
966	Glucosinolate biosynthesis	M00370	C05837	Glucobrassicin	
311	Penicillin biosynthesis	M00672	C00395	Penicillin	beta-Lactam antibiotic
	Cephalosporin biosynthesis	M00673	C06566	Cephamycin C	
332	Carbapenem biosynthesis	M00675	C06669	Carbapenem-3-carboxylate	
261	Monobctam biosynthesis	M00736	C01941	Nocardicin A	
331	Clavaminic acid biosynthesis	M00674	C06660	Clavaminic acid	
521	Streptomycin biosynthesis	M00793	C00413	Streptomycin	Aminoglycoside antibiotic
524	Kanamycin biosynthesis		C01822	Kanamycin A	
			C00825	Kanamycin B	
			C01823	Kanamycin C	
	Neomycin biosynthesis		C01441	Neomycin A	
			C01737	Neomycin B	
			C15652	Neomycin C	
	Gentamicin biosynthesis		C07656	Gentamicin C1	
			C00908	Gentamicin C1a	
			C02033	Gentamicin C2	
			C17706	Gentamicin C2b	
	Paromomycin biosynthesis		C00832	Paromomycin	Aminoglycoside antibiotic, antiparasitic
525	Acarbose biosynthesis	M00814	C06802	Acarbose	C7N-aminocyclitol, antidiabetic drug
	Validamycin biosynthesis	M00815	C12112	Validamycin A	C7N-aminocyclitol antibiotic, fungicide
			C21293	Validamycin B	
401	Novobiocin biosynthesis		C05080	Novobiocin	Aminocoumarin antibiotic
	Coumermycin biosynthesis		C05073	Coumermycin A1	

	Clorobiocin biosynthesis		C12032	Clorobiocin	
404	Staurosporine biosynthesis	M00805	C02079	Staurosporine	Indolocarbazole alkaloid, PKC inhibitor
	Rebeccamycin biosynthesis	M00789	C19701	Rebeccamycin	Indolocarbazole alkaloid, antitumor antibiotic
	Violacein biosynthesis	M00808	C21136	Violacein	Bisindole alkaloid
	Pyrrolnitrin biosynthesis	M00790	C12491	Pyrrolnitrin	Pyrrole antibiotic
	Fumitremorgin alkaloid biosynthesis	M00786	C20564	Fumitremorgin A	Mycotoxin
	Meleagrin biosynthesis		C22168	Meleagrin	
405	Phenazine biosynthesis		C21476	Phenazine	Phenazine
	Pyocyanine biosynthesis	M00835	C01748	Pyocyanine	
	PQS biosynthesis		C11848	2-Heptyl-3-hydroxy-quinolone	Quinolone
			C20643	2-Heptyl-4-quinolone	
333	Prodigiosin biosynthesis	M00837	C21565	Prodigiosin	Tripyrrole antibiotic
	Undecylprodigiosin biosynthesis	M00838	C12023	Undecylprodigiosin	
254	Aflatoxin biosynthesis	M00937	C06800	Aflatoxin B1	Mycotoxin
996	Cucurbitacin biosynthesis		C08794	Cucurbitacin B	Triterpenoid
			C08795	Cucurbitacin C	
			C08797	Cucurbitacin E	
	Solanine and tomatine biosynthesis		C10820	Solanine	Steroid alkaloid
			C10827	Tomatine	
	Ephedrine biosynthesis		C01575	Ephedrine	Phenethylamine alkaloid
			C02765	Pseudoephedrine	
	Capsaicin biosynthesis		C06866	Capsaicin	Capsaicinoid/vanilloid, highly pungent
			C00755	Vanillin	Vanilloid, vanilla flavoring
	Acridone alkaloid biosynthesis		C10632	Acronycine	
			C10722	Melicopicine	
			C10738	Rutacridone	
	(Quinoline alkaloid)		C01897	Camptothecin	Quinoline alkaloid
			C11379	Cinchonidine	
			C06528	Cinchonine	
998	Kanosamine biosynthesis	M00877	C12212	Kanosamine	Aminosugar antibiotic
	Aurachin biosynthesis	M00848	C21875	Aurachin A	Quinoline antibiotic
			C21140	Aurachin B	
			C21330	Aurachin C	
			C10641	Aurachin D	
	Bacilysin biosynthesis	M00787	C20942	Bacilysin	Dapdiamide antibiotic
	Dapdiamides biosynthesis	M00904	C20962	Dapdiamide A	
			C20963	Dapdiamide B	
			C20964	Dapdiamide C	
	Puromycin biosynthesis	M00889	C01610	Puromycin	Aminonucleoside antibiotic
	Fosfomycin biosynthesis	M00903	C06454	Fosfomycin	Phosphonate antibiotic
	Cremeomycin biosynthesis	M00951	C22416	Cremeomycin	ortho-Diazoquinone, cytotoxic antibiotic
	Fumagillin biosynthesis	M00969	C09668	Fumagillin	Meroterpenoid antibiotic
	Pentalenolactone biosynthesis	M00819	C20407	Pentalenolactone	Sesquiterpenoid antibiotic
	Terpentecin biosynthesis	M00788	C21093	Terpentecin	Diterpenoid antibiotic
	Roseoflavin biosynthesis	M00890	C21647	Roseoflavin	Riboflavin analogue
	Cycloserine biosynthesis	M00785	C08057	Cycloserine	Alanine analogue antibiotic

999	Crocin biosynthesis	M00900	C08589	Crocin	Apocarotenoid
			C08588	Crocetin	
	Ginsenoside biosynthesis		C20713	Ginsenoside Rb1	Triterpene saponin
			C08946	Ginsenoside Rg1	
	Saponin adjuvant biosynthesis	M00971	C22666	QS-7	
	Cannabidiol biosynthesis	M00894	C07578	Cannabidiol	Cannabinoid
			C06972	Dronabinol	
	Mugineic acid biosynthesis	M00953	C15500	Mugineic acid	Phytosiderophore
	Pentagalloylglucose biosynthesis		C04576	Pentagalloylglucose	Gallotannin/ellagitannin precursor
	Benzoxazinoid biosynthesis	M00952	C15770	DIBOA	Benzoxazinoid, plant antibiotic, defense compound
			C15772	DIBOA-glucoside	
			C04831	DIMBOA-glucoside	
	Gramine biosynthesis		C08304	Gramine	Indole alkaloid
	Coumarin biosynthesis		C05851	Coumarin	Coumarin
999	Furanocoumarin biosynthesis		C09315	Umbelliferone	Furanocoumarin
			C09305	Psoralen	
			C22152	Bergamottin	
	Hordatine biosynthesis		C08307	Hordatine A	Hordatine, defense compound
			C08308	Hordatine B	
	Podophyllotoxin biosynthesis	M00902	C10874	Podophyllotoxin	Lignan
997	Ditryptophenaline biosynthesis	M00891	C22144	(-)-Ditryptophenaline	Indole alkaloid
	Fumiquinazoline D biosynthesis	M00901	C22149	Fumiquinazoline D	Mycotoxin
	Paerucumarin biosynthesis		C22449	Paerucumarin	Coumarin
	Staphyloferrin A biosynthesis	M00876	C22102	Staphyloferrin A	Siderophore
	Staphyloferrin B biosynthesis	M00875	C22075	Staphyloferrin B	
	Aerobactin biosynthesis	M00918	C05554	Aerobactin	
	Cyclooctatin biosynthesis	M00921	C21979	Cyclooctatin	Diterpenoid, lysophospholipase inhibitor
	Lovastatin biosynthesis	M00893	C21130	Lovastatin acid	Fungal polyketide
	Grixazone biosynthesis	M00905	C20799	Grixazone B	Grixazone, yellow pigment
	Ethynylserine biosynthesis	M00906	C22141	L-β-Ethynylserine	Non-proteinogenic amino acid

6 FINAL CONSIDERATIONS

This thesis highlighted findings about the importance of *Streptomyces* spp. reported as a source of new antimicrobial compounds and highlighting the need of exploiting microbial biodiversity for biotechnological applications. Further results showed the activation of biosynthetic genes and the utilization of metabolic profiles for the development of innovative strategies to combat antimicrobial resistance.

In this scenario, Chapter 1 showed an extensive scoping review about new and old compounds isolated from environmental bacteria. The genus *Streptomyces* was the most interesting regarding amplitude of activity against the ESKAPEE pathogens, in addition to, isolation from marine environments (like sea sponges, sediments, algae) showed undercover antibiotics, that can be used in the future due to its large applicability.

In the chapter 2, was presented the high incidence of antimicrobial resistance in clinical bacteria isolated in the Clinical Hospital of the Federal University of Paraná. The resistance genes were detected in isolates of *Klebsiella* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Escherichia coli*, *Enterobacter cloacae*, *Enterococcus* spp. and isolates of *Staphylococcus aureus* (all together these species are called ESKAPEE pathogens). *Bacterial resistance* represents a challenge to clinicians owing to the difficulty in treating infection, and the high prevalence and mortality, which increase the need to find new compounds that can overtake the resistant bacteria.

Furthermore, in chapters 3 and 4, this thesis investigates the antibacterial activity of marine *Streptomyces* isolates from Ilha do Mel, Parana coast. These strains were all deposited in Microbiological Collections Centre of the Taxonline Network of Paraná (CMRP/Taxonline) The Paraná coast have an unexplored biotechnological potential, and these actinobacteria isolated in the Ilha do Mel, showed good activity against the ESKAPEE pathogens. In special, the strain CMRP6046, identified as *Streptomyces cavourensis*, showed high activity against MDR bacteria, and its genome was processed to evaluate metabolites routes that can be related to the production of the compounds. The genome exhibited a rich repertoire of 32 putative secondary metabolite biosynthetic gene clusters (BGCs), encompassing RiPPs, PKS, NRPS, terpenes, siderophores, melanin, ectoine, and butyrolactone, and combining these results with future mass spectrometry analysis, can indicate the new potential antibiotics.

This thesis helps to consolidate a large microbiological collection deposited Microbiological Collections Centre of the Taxonline Network of Paraná (CMRP/Taxonline). The study prospected a potential antibiotic producers isolated from Brazilian environment. In addition, the CMRP/Taxonline collection also has several clinical strains with MDR from the Paraná state, and this thesis is the first one to largely investigate the extent of resistant in the state, which can help public health authorities combat MDR.

A comprehensive chromatographic investigation of the secondary metabolites of *S. cavourensis* CMRP6046, encompassing flavonoids, phenolic acids, alkaloids, terpenoids, and other classes of compounds, is currently underway. Subsequent studies will focus on the fractionation and purification of bioactive compounds, as well as the quantitative determination of safe concentrations that can be employed to enhance existing drugs or yield new bioproducts against MDR bacteria.

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