UNIVERSIDADE FEDERAL DE VIÇOSA

# Bioprospection and adaptive mechanisms of halotolerant bacteria: applications in biotechnology and astrobiological studies

Camila de Souza Vieira
*Magister Scientiae*

VIÇOSA - MINAS GERAIS
2025

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**Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa**

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Vieira, Camila de Souza, 1998-

V658b
2025

Bioprospection and adaptive mechanisms of halotolerant bacteria: applications in biotechnology and astrobiological studies / Camila de Souza Vieira. – Viçosa, MG, 2025.

1 dissertação eletrônica (93 f.): il. (algumas color.).

Texto em inglês.

Orientador: Marcos Rogério Tótola.

Dissertação (mestrado) - Universidade Federal de Viçosa, Departamento de Microbiologia, 2025.

Inclui bibliografia.

DOI: https://doi.org/10.47328/ufvbbt.2025.247

Modo de acesso: World Wide Web.

1. Extremófilos. 2. Exobiologia. 3. Tolerância a sal. I. Tótola, Marcos Rogério, 1965-. II. Universidade Federal de Viçosa. Departamento de Microbiologia. Programa de Pós-Graduação em Microbiologia Agrícola. III. Título.

CDD 22. ed. 578.758

Bibliotecário(a) responsável: Bruna Silva CRB-6/2552

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APPROVED: February 24, 2025.

Assent:

Camila de Souza Vieira
Author

Marcos Rogerio Totola
Adviser

# ACKNOWLEDGMENTS

This journey would never be the same without love—the love I have for science and the love I give and receive from the people around me.

To God and companionable spirituality.

To my parents, Sergio and Claudia, you are my fortress and foundation in this world. Thank you for dreaming with me and teaching me that education is always the best path.

To my heart family: Julia, Iury, Victória, Pablo, and Mirelly. Thank you for choosing me.

To my lab partners: Mariana, Jéssica, Caio, Thiago, Jomar and Ana. You brought light to my days.

To Professor Edmo Montes, for their invaluable contribution to this work.

To Letícia, for all the coffees and for going above and beyond your job.

To Alex, for all contributions and advice since the beginning of my scientific career.

To my advisor, Marcos Tótola. Thank you for believing in and supporting my dreams, even the Martian ones. This certainly changed my life.

To Universidade Federal de Viçosa, for all the opportunities and realizations.

This work has been sponsored by the following Brazilian research agencies: Coordination for the Improvement of Higher Education Personnel (CAPES; Financing code 001), Minas Gerais State Foundation for Research Aid (FAPEMIG) and National Council of Scientific and Technological Development (CNPq).

# ABSTRACT

VIEIRA, Camila de Souza, M.Sc., Universidade Federal de Viçosa, February, 2025. **Bioprospection and adaptive mechanisms of halotolerant bacteria: applications in biotechnology and astrobiological studies**. Adviser: Marcos Rogerio Totola.

Halophilic and halotolerant bacteria can be applied in environments where conventional organisms cannot thrive, such as in the bioremediation of salinized soils or the treatment of saline effluents. These organisms are also frequently used in astrobiology studies due to their well-recognized ability to tolerate or adapt to extreme conditions. Brazil, with its diverse biomes and high biodiversity, harbors saline environments that remain largely unexplored in terms of microbial biodiversity. This study aimed to investigate the biotechnological potential and the feasibility of using halotolerant bacteria isolated from extreme Brazilian environments (arid and saline) as model organisms in astrobiological studies, as well as to expand the understanding of the adaptive capacity of halotolerant microorganisms to NaCl. The bacteria were collected from three areas in Camocim, Ceará, Brazil. Their identification was carried out through fatty acid methyl ester (FAME) profiling and the analysis of the DNA sequence encoding the 16S rRNA. The requirement for or tolerance to NaCl and sodium perchlorate ($NaClO_4$) were evaluated by determining the specific growth rates (μ) in culture media containing different salt concentrations. The ability to use $NaClO_4$ as a final electron acceptor was also assessed. The biotechnological potential of the isolates was evaluated by analyzing their production of biosurfactants, exopolysaccharides, and the enzymes lipase, protease, and amylase. To study the adaptive capacity of microorganisms to sodium chloride concentrations above the maximum originally tolerated by the wild-type strain, the technique of adaptive laboratory evolution (ALE) was applied. The study used one of the isolates collected in Camocim, selected based on its μ value at 100 g $L^{-1}$ of NaCl, and a bacterium previously isolated from Trindade Island, Brazil, which had demonstrated high adaptability to NaCl. The evolved and parental/wild-type strains were compared using growth curves and specific growth rate values at different NaCl concentrations. Additionally, it was assessed whether the mutations generated by ALE altered the growth rate in the presence of $NaClO_4$ as a stressor and whether they affected the fatty acid profile in response to NaCl concentration in the growth medium. Among the isolates, those belonging to the genus Bacillus were predominant (18/20). All isolates were able to grow at 100 g $L^{-1}$ NaCl and at least 30 g $L^{-1}$ $NaClO_4$, in addition to being able to reduce $NaClO_4$ in the absence of other electron acceptors. The isolates could produce biosurfactants, exopolysaccharides, and enzymes of industrial/biotechnological interest. ALE resulted in variants with a higher specific growth rate and greater tolerance to higher NaCl concentrations compared to the parental strains. Furthermore, an increase in μ at 70 g $L^{-1}$ $NaClO_4$ was observed, along with changes in the fatty acid profile under both saline and non-saline stress conditions.

Keywords: extremophiles; astrobiology; salt adaptation; adaptive laboratory evolution

# RESUMO

VIEIRA, Camila de Souza, M.Sc., Universidade Federal de Viçosa, fevereiro de 2025. **Bioprospecção e mecanismos adaptativos de bactérias halotolerantes: aplicações na biotecnologia e em estudos astrobiológicos**. Orientador: Marcos Rogerio Totola.

Bactérias halofílicas e halotolerantes podem ser aplicadas em áreas onde organismos convencionais não conseguem atuar, como por exemplo na biorremediação de solos salinizados ou no tratamento de efluentes salinos. Esses organismos são também utilizados com frequência em estudos de astrobiologia, dada a sua reconhecida capacidade de tolerar ou adaptar-se a condições extremas. O Brasil, com sua diversidade de biomas e elevada biodiversidade, abriga ambientes salinos pouco explorados no que concerne à biodiversidade microbiana. Este trabalho teve como principais objetivos estudar o potencial biotecnológico e a possibilidade de se utilizarem bactérias halotolerantes isoladas de ambientes extremos brasileiros (áridos e salinos) como organismos-modelo em estudos astrobiológicos, bem como ampliar o entendimento da capacidade de adaptação de microrganismos halotolerantes ao NaCl. As bactérias foram coletadas em 3 áreas de Camocim, Ceará, Brasil. Sua identificação foi feita por meio da análise dos perfis de ésteres metílicos de ácidos graxos (FAME) e pela análise da sequência de DNA que codifica para o rRNA 16S. A exigência de ou tolerância ao NaCl e ao perclorato de sódio (NaClO4) foram avaliadas por meio da determinação das taxas específicas de crescimento (µ) em meios de cultura contendo diferentes concentrações dos sais. Também foi avaliada a capacidade de utilização do $NaClO_4$ como aceptor final de elétrons. O potencial biotecnológico dos isolados foi avaliado a partir da análise da produção de biossurfactantes, exopolissacarídeos e das enzimas lipase, protease e amilase. Para estudar a capacidade de adaptação dos microrganismos a concentrações de cloreto de sódio acima da máxima tolerada originalmente pela linhagem selvagem, foi utilizada a técnica de evolução adaptativa em laboratório (EAL). Utilizou-se no estudo um dos isolados coletados em Camocim, selecionado com base no valor de µ a 100 g $L^{-1}$ de NaCl, e uma bactéria isolada em trabalhos anteriores na Ilha da Trindade, Brasil, que demonstrou alta adaptabilidade ao NaCl. As linhagens evoluídas e parentais/selvagens foram comparadas por meio de curvas de crescimento e pelos valores de taxa específica de crescimento em diferentes concentrações de NaCl. Adicionalmente, avaliou-se se as mutações geradas pela EAL alteraram a taxa de crescimento na presença de $NaClO_4$ como agente estressor e o perfil de ácidos graxos em resposta à concentração de NaCl no meio de crescimento. Dentre os isolados, predominaram aquelas pertencentes ao gênero Bacillus (18/20). Todos os isolados cresceram em 100 g $L^{-1}$ de NaCl e em pelo menos 30 g $L^{-1}$ de $NaClO_4$, além de serem capazes de reduzir $NaClO_4$ na ausência de outros aceptores de elétrons. Os isolados foram capazes de produzir biossurfactantes, exopolissacarídeos e enzimas de interesse industrial/biotecnológico. A EAL resultou na obtenção de variantes que apresentaram maior taxa de crescimento específico e tolerância a maiores concentrações de NaCl comparativamente às linhagens parentais. Observou-se ainda aumento de µ a 70 g $L^{-1}$ de $NaClO_4$ e alterações no perfil de ácidos graxos com e sem estresse salino.

Palavras-chave: extremófilos; astrobiologia; adaptação a sal; evolução adaptativa em laboratório

## Summary

# INTRODUÇÃO GERAL

Organismos extremófilos podem ser aplicados em diversos setores da economia, como nas indústrias de detergente, de papel, têxtil e de biocombustíveis, assim como na biorremediação de ambientes contaminados, tratamento de águas residuárias, na agricultura e na astrobiologia (Atuchin *et al.*, 2023; Demain e Adrio, 2007; Joshi *et al.*, 2023; Ramasamy e Mahawar, 2023; Schultz *et al.*, 2023; Tan *et al.*, 2019). Dentre os extremófilos, os halofílicos e halotolerantes são aplicáveis tanto na biotecnologia quanto na astrobiologia (Bryanskaya *et al.*, 2019; Dutta e Bandopadhyay, 2022; Santos *et al.*, 2023; stan-lotter, 2019). Microrganismos halofílicos necessitam de cloreto de sódio (NaCl) para sobreviver, enquanto halotolerantes toleram concentrações deste sal no meio (Larsen, 1986). Estes organismos são capazes não só de viver em meio com NaCl, o que os caracteriza como halófilos/halotolerantes, mas também em meios ricos em outros sais, como os de perclorato e sulfato (Bryanskaya *et al.*, 2013). No Brasil, microrganismos halofílicos e halotolerantes podem ser encontrados em ambientes ricos em sal como no bioma de mangues, na lagoa de Araruama no Rio de Janeiro e nas salinas naturais no Rio Grande do Norte e no Ceará. Entretanto, estes locais foram pouco explorados quanto à composição e caracterização de seu microbioma, especialmente quando comparados a outros locais salinos no mundo, como o Mar Morto e o Grande Lago de Sal (Camara, 2021; Cardoso *et al.*, 2011; Dassarma, 2006; Diniz e Vasconcelos, 2016; Duarte *et al.*, 2018).

Microrganismos halofílicos e halotolerantes são, em especial, aplicáveis no tratamento de águas residuárias ricas em sal. O tratamento biológico de águas residuárias é econômico e pouco (ou nada) danoso ao ambiente. Contudo, a salinidade das águas residuárias de alguns setores, como o de couro, pode ser um empecilho para o tratamento biológico. Normalmente, as empresas contornam este problema evaporando a água e deixando o sal no fundo, porém este processo é caro e laborioso. Neste contexto, o tratamento biológico de águas residuárias Salinas por halofílicos/halotolerantes e/ou suas enzimas é uma solução mais simples, econômica e ambientalmente favorável (Ariaeenejad *et al.,*2023; Tan *et al.*, 2019). A maior parte das águas residuárias são formadas por polissacarídeos, lipídeos e proteínas, de modo que a produção de amilases, lipases e proteases por microrganismos halofílicos/halotolerantes é um alvo na bioprospecção de organismos aplicáveis ao seu tratamento (Linarić *et al.*, 2013). Adicionalmente, microrganismos halofílicos/halotolerantes também podem ser usados na recuperação avançada de petróleo e na biorremediação de ambientes contaminados com óleo e água de produção salina. Nestes dois processos, a produção de biossurfactantes tolerantes a condições de salinidade elevada é uma opção biotecnológica relevante, dada a capacidade desses compostos de solubilizar os hidrocarbonetos (biorremediação) e de reduzir a tensão entre o óleo e a água de injeção (recuperação avançada de petróleo) (Somee *et al.*, 2021).

Microrganismos halofílicos/halotolerantes são relevantes também em estudos de astrobiologia (Emlik & Marakli, 2023). Marte, nosso vizinho mais próximo, possui os elementos principais e elementos traços importantes para a manutenção da vida. Além disso, é comprovado que já houve água líquida durante o período Noachian e, atualmente, existem indícios da existência de água líquida subterrânea na forma de salinas (Cockel, 2014). Nesse planeta, o perclorato presente na superfície e possivelmente nas prováveis salinas subsuperficiais é visto como um empecilho à vida (Oren *et al.*, 2013). Este sal é altamente oxidante e é provável que o efeito inibitório que apresenta sobre os organismos vivos seja devido ao estresse oxidativo, ao qual microrganismos halofílicos tendem a ser resistentes (Laye and Dassarma, 2018). Alguns estudos mostram que organismos halofílicos são capazes de sobreviver em concentrações de perclorato maiores do que as detectadas em Marte (4-6 g $L^{-1}$) (Flores *et al.*, 2020; García-Descalzo, 2023). Diferentemente de Marte, as luas de gelo Enceladus e Europa possuem como principal sal o NaCl, em concentrações que podem variar dependendo da localização no oceano salino que abrigam (Ashkenazy e Tziperman, 2021; Trumbo *et al.*, 2022; Weber *et al.*, 2023). Além de água líquida, Enceladus possui outros elementos importantes à vida (Kang *et al.*, 2022) e ambas as luas são geologicamente ativas (Weber *et al.*, 2023).

Diante de todas estas aplicabilidades, a compreensão dos mecanismos adaptativos de microrganismos halofílicos e halotolerantes mostra-se como sendo de elevada importância, tanto para se aumentar a aplicabilidade de microrganismos na biotecnologia, quanto para ampliar o entendimento dos limites da vida. Uma forma de se entenderem os mecanismos adaptativos destes microrganismos é justamente simulando a evolução darwiniana em laboratório, em um processo chamado Evolução Adaptativa em Laboratório (EAL) e aplicando técnicas de sequenciamento genômico completo (Barrios, 2013). Outro mecanismo adaptativo e que é uma resposta fisiológica relevante é a alteração no perfil de ácidos graxos de membrana, o que pode contribuir para uma maior resistência ao estresse ambiental (Wang *et al.*, 2020). Deste modo, o presente trabalho objetiva demonstrar o potencial biotecnológico e para estudos astrobiológicos de bactérias halotolerantes isoladas de ambientes brasileiros, bem como ampliar o entendimento da adaptação de microrganismos halotolerantes a altas concentrações de NaCl por meio da aplicação da EAL.

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# CHAPTER I: HALOPHILES AND HALOTOLERANTS: FROM INDUSTRY TO ASTROBIOLOGY

## INTRODUCTION

Extremophiles have gained significant prominence in recent years due to their unique ability to survive in harsh environments. They were originally defined by Macelroy (1974) as "organisms able to populate environments hostile to mesophiles, or organisms which grow only in intermediate environments”. Extremophile microorganisms can be applied in several industrial sectors such as food, medicine, textile, leather, detergent, and biofuel as well as in agriculture and bioremediation (Atuchin *et al.* 2023; Demain e Adrio. 2007; Joshi *et al.* 2023; Ramasamy e Mahawar. 2023; Tan *et al.* 2019). Furthermore, extremophiles are models for astrobiological studies because these organisms can live on Earth in environments similar to those found on other planets and moons. (Seckbach & Stan-Lotter 2020).

Extremophiles can be classified based on their ability to survive in different environments. Thermophiles and hyperthermophiles can tolerate high temperatures, while psychrophiles and psychrotolerants can survive at low temperatures. Acidophiles and alkalophilic organisms can survive in low and high pH environments, respectively. There are also barophilic organisms that can thrive under high pressures and halophiles and halotolerant organisms that can survive in high concentrations of sodium chloride (NaCl) (Ali *et al.* 2023). Table 1 presents some examples of extremophiles, including representatives from the Eukarya, Archaea, and Bacteria domains and their potential applications.

Halophiles need NaCl to live. According to the salt concentration required for growth, they are classified as slight (20 to 30 g $L^{-1}$), moderate (50 to 200 g $L^{-1}$), and extreme halophiles (200 g $L^{-1}$). On the other hand, halotolerants are microorganisms that do not require high salt concentration for growth, but withstand high NaCl concentrations. Accordingly, they are classified as slight (growth in salt concentration up to 60 to 80 g $L^{-1}$), moderate (180 to 200 g $L^{-1)}$, and extreme halotolerants (up to the salt saturation) (Larsen, 1986).

Halophiles and halotolerants have applications in industry and biotechnology. They can be used in wastewater biological treatment (Zaerva *et al.* 2021), and bioremediation of petroleum-contaminated sites (Ghaedrahmat *et al.* 2022). These microorganisms are particularly useful in contaminated environments with high salt concentrations, since commonly used microorganisms cannot survive and thrive in such environments (Zhao *et al.* 2023). Furthermore, they have potential applications in astrobiology, since they could survive in the salty oceans on the ice moons Europa and Enceladus and in the perchlorate brines of Mars (Bryanskaya *et al.* 2013; Cockell 2014; Jebbar *et al.* 2020; Trumbo *et al.* 2022; Weber *et* *al.* 2023).

This review provides an overview of the primary applications of halophile and halotolerant microorganisms in industry and biotechnology. It also provides insights into the potential applications of these microorganisms in astrobiology and covers articles that have studied halophiles and halotolerants for astrobiological purposes. Finally, the article highlights the importance of understanding the mechanisms that enable halophiles and halotolerants to survive in extreme environments.

**Table 1** Examples of extremophiles of the three domains of life isolated from various sites around the world and their classification and possible applications.

| **Strain** | **Domain** | **Site** | **Classification** | **Potential applications** | **Reference** |
|---|---|---|---|---|---|
| *Meyerozyma guilliermondii*, *Penicillium chrysogenum, P. cf. palitans, Pseudeurotium cf. bakeri, Thelebolus balaustiformis, Antarctomyces psychrotrophicus, and Cladosporium sp.* | Eukarya | South Shetland Islands, maritime Antarctica | Psychrotolerant and psychrophilic | Production of hydrolytic enzymes, biosurfactants and phytotoxic metabolites | da Silva *et al.* 2024 |
| *Pyrococcus* sp. strain M24D13 | Archaeae | Hydrothermal site of Deception Island, Antarctica | Hyperthermophilic | Astrobiology study of icy worlds | Uribe-Redlich *et al.* 2024 |
| *Halovibrio variabilis TG-5* | Bacteria | Crystallization pool of a solar saltern in Tianjin, China. | Halophilic (Optimum of 150 g $L^{-1}$) and thermophilic (optimum of 46°C) | Pretreatment of coal gasification wastewater | Sun *et al.* 2024 |
| *Thalassobacillus sp.* | Bacteria | Hypersaline lakes, saline soils, salt flats, and volcanic mud | Moderately halophilic (1-150 g $L^{-1}$) | Bioremediation, enzymes, biosurfactants, and biofuels production | Tuesta-Popolizio *et al.* 2021 |
| *Schwanniomyces etchellsii* | Eukarya | Urban city botanical garden, Bangkok, Thailand | Acid-thermotolerant | Host for commercial bio-based production | Lertsriwong *et al.* 2023 |
| *Alteromonas sp. ORB2* | Bacteria | Halophilic aerobic granular sludge | Halophilic (30,5-90,5 g $L^{-1}$) alkaliphilic (pH 5-11) | Use in the bioremediation of Cr (VI)-containing wastes | Reddy *et al.* 2024 |

## ADAPTIVE MECHANISMS OF HALOPHILES AND HALOTOLERANTS

Some adaptive mechanisms allow halophiles and halotolerants to withstand harsh conditions. Non halophiles/halotolerants, when exposed to high salt concentration, lose water from their cells by osmosis. On the other hand, halophiles and halotolerants protect themselves by salt-in and salt-out mechanisms and adaptation of the enzymatic machinery (Mukhtar *et al.*

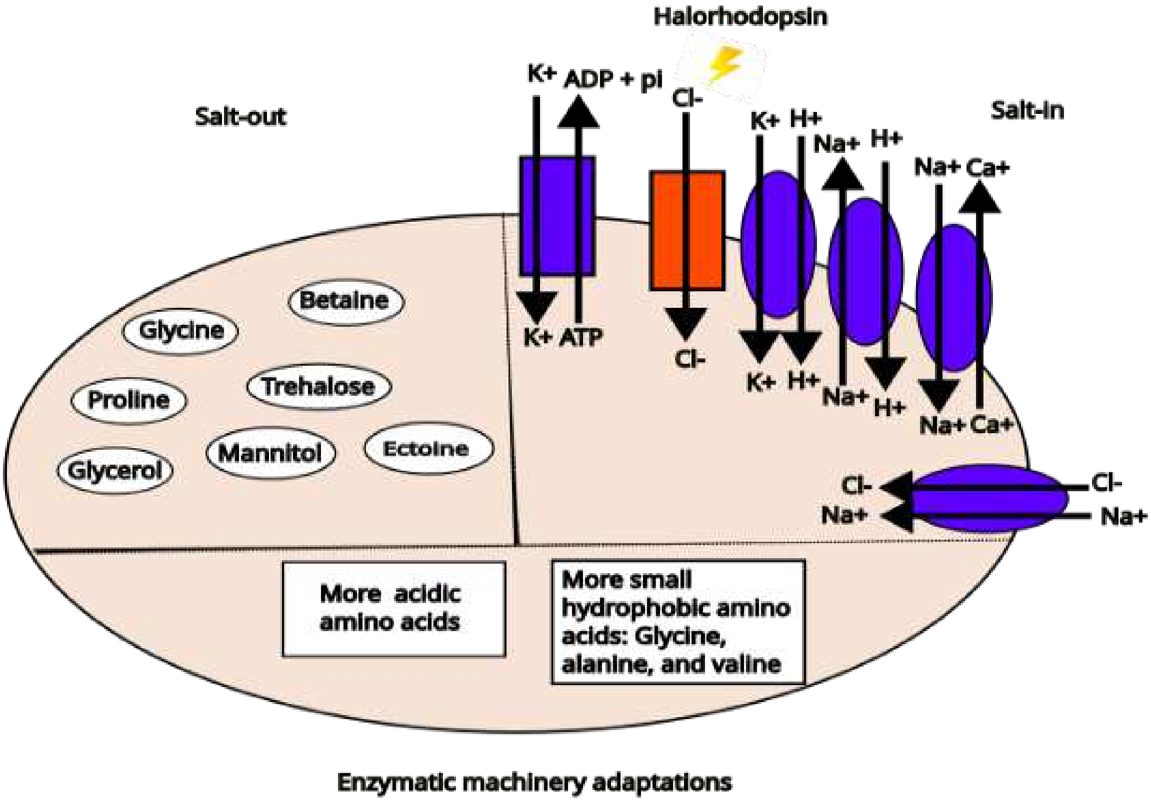
2020). Salt-in refers to the maintenance of the ionic concentration in the cell. This maintence is achieved by $K^+$ uptake using passive transport channels and active transport systems, by $H^+/K^+$ symporter, and by $H^+/Na^+$ and $Ca^+/Na^+$ antiporter. $Na^+$ is more harmful to the cell than $K^+$, so changing $K^+$ for $Na^+$ is a common strategy for survival in saline environments. Salt-in strategies also include $Cl^-$ uptake by light dependent and independent strategies, as reported in *Halobacteriaceae*. Light dependent $Cl^-$ uptake occurs through chloride pumps mediated by halorhodopsin, while light-independent $Cl^-$ uptake is mediated by $Cl^-/Na^+$ symporters. Cell functions of chloride-dependent microorganisms, such as the halophilic bacteria *Halobacillus halophilus*, are only expressed in the presence of this anion. These include the uptake of glycine betaine, a compatible solute used in salt-out strategy (DasSarma and Arora 2001; Gunde-Cimerman & Oren 2018; Saini *et al.* 2023; Saum & Müller 2008) (Fig.1).

Salt-out involves the accumulation of compatible solutes such as betaine, proline, ectoine, glycine, glycerol, trehalose, and mannitol that can be synthesized or uptake from the medium. Salt-out keeps the osmotic pressure and avoids loss of water for the medium, and also stabilizes proteins. In high salt concentrations, osmolytes are excluded from the protein surface due to competition with water, which have a more favorable interaction with proteins and bonds to the protein surface, making them hydrated. Additionally, the interaction with osmolytes increases the protein free energy and folds them (Jadhav *et al.* 2018; Saini *et al.* 2023) (Fig.1).

Srivastava et al. (2022) conducted transcriptomic analyses of the halophilic bacterium *Chromohalobacter salexigens* ANJ207 at different NaCl concentrations to understand the mechanisms involved in salt stress adaptation. At 100 g $L^{-1}$ NaCl, there was an upregulation of genes encoding lactate dehydrogenase and catalase, while at 250 g $L^{-1}$ NaCl, there was an increase in genes encoding polynucleotide phosphatase, potassium transporters, aconitase, the ABC subunit of excinuclease, and transposase. Additionally, real-time quantitative PCR showed that genes related to glycine betaine biosynthesis and uptake were upregulated, especially at 100 g $L^{-1}$. Similar to *C. salexigens* ANJ207, the halotolerant bacterium *Egicoccus halophilus* EGI 80432T also exhibited increased expression of genes related to the synthesis of compatible solutes, including glutamate, proline, histidine, ectoine, and threonine (Chen et al., 2021).

In addition to the salt in and salt out mechanisms, halotolerant/halophilic microorganisms have other forms of adaptation to high salinity. Halophilic enzymes have a unique composition compared to nonhalophilic enzymes. They contain more acidic amino acids, such as aspartate and glutamate. This configuration forms a network of hydrated salts that prevents protein aggregation. In addition, halophilic enzymes have higher concentrations of hydrophobic residues (glycine, alanine, and valine) and more ionic bonds at specific sites, a characteristic that is also observed in thermophilic enzymes and that can explain the polyextremophile character of halophilic enzymes. These enzymes also present a lower proportion of aliphatic residues (leucine and isoleucine) (Madern *et al.* 1995; Munawar & Engel 2013; Sengupta *et al.* 2023) (Fig.1).

**Fig. 1** Adaptive mechanisms of halophilic microorganisms



## HALOPHILIC ENZYMES

The enzymes market has been on a steady increase over the years, and it's expected to continue growing in the coming years. According to Markets and Market (2024), the global enzymes market size was $7.2 billion in 2023 and is expected to reach $10.2 billion by 2028, with a compound annual growth rate (CAGR) of 6.6%.

Enzymes are widely used in various industries such as detergent, food, pharmaceutical, and biofuel due to their technical, economic, and environmental benefits over chemical catalysts. However, certain industrial conditions pose a challenge to mesophile enzymes, such as high and low temperatures, toxic solvents, extreme pH levels, high pressures, and high salinities (Chapman *et al.* 2018; Littlechild 2015). One solution to this problem is to use enzymes from extremophile microorganisms for biocatalysis in harsh industrial conditions (Arya *et al.* 2022). Halophilic enzymes, for instance, can withstand high concentrations of NaCl and maintain their activity in other extreme conditions such as high temperatures and pH levels, making them polyextremophiles (Delgado-García *et al.* 2012).

De Lourdes Moreno *et al.* (2013) pointed out that halophilic enzymes have the potential to be a novel catalyst alternative for various industries. However, they are still poorly studied, and only a few applications have been reported. While lipases can be used in the industry due to their catalytic diversity, such as hydrolysis of triacylglycerols, esterification reactions, transesterification, alcoholysis, and acidolysis, only a few of them are known to withstand industrial conditions. In this regard, the polyextremophilic character of halophilic lipases is a desirable characteristic that can increase the utilization of lipases in the industry. Therefore, it is crucial to study halophilic and halotolerant microorganisms as a source of lipases (Qiu *et al.* 2021).

As well as lipases, proteases from halophiles are polyextremophilics, as they can remain active in a wide range of pH and temperature. This adaptability renders them highly versatile and desirable for numerous industrial applications (Mokashe *et al.* 2018). Additionally, other halophilic enzymes such as amylase, xylanase, and laccase have been reported to have industrial applications (Mesbah *et al.* 2022).

Studies have identified halophiles and halotolerants from various locations and examined their ability to produce enzymes. Mukhtar *et al.* (2019) found that microorganisms isolated from the Khewra salt mine in Pakistan could produce protease, lipase, catalase, amylase, urease, cellulase, and gelatinase. Gupta *et al.* (2016) analyzed the enzyme production of halophilic bacteria from soil sediment in Himachal Pradesh, India, and found they could produce amylase, protease, lipase and glutaminase. In another study, rhizospheric and endophytic bacteria from seven mangroves in Saudi Arabia were found to produce amylase, proteases, and lipases (Bibi *et al.* 2017). Finally, bacteria from the Atacama Desert were found to produce lipases, pectinases, and cellulases (Ruginescu *et al.* 2019).

## WASTEWATER TREATMENT

Halophilic enzymes offer a promising solution for wastewater treatment, particularly in industries where high salt content poses a challenge to conventional biological treatment systems. Desalination processes such as mechanical vapor compression (MVC), membrane distillation (MD) and forward osmosis have been usually applied to allow wastewater reuse. These methods along with coagulation and flocculation process can also be employed as pre-treatment steps before wastewater treatment in MBR. Despite being effective, these pre-treatment techniques are expensive and laborious (Tan *et al.* 2019). In contrast, utilizing halophilic/halotolerant hydrolases for the treatment of saline wastewater is both cost-effective and eco-friendly (Ariaeenejad *et al.*2023).

For instance, Pounsamy et al. (2019) demonstrated the efficacy of immobilized halotolerant lipase from *Bacillus cereus* isolated from soak liquor in removing fat from saline wastewater in the leather industry. Similarly, halotolerant amylase from *Providencia rettgeri*, and proteases from *Proteus mirabilis, Pseudomonas aeruginosa*, and *Enterococcus faecalis* - all bacteria isolated from saline tannery wastewater - have shown promise in degrading starch and proteins, respectively, in saline tannery wastewater (Maharaja et al. 2017; Maharaja et al. 2018; Maharaja et al. 2019; Sivaprakasam et al. 2011). Additionally, Ali et al. (2014) found that amylase from halophilic *Aspergillus gracilis*, isolated from a man-made saltern in Thailand, outperforms commercial counterparts, displaying higher activity across various salt concentrations in simulated wastewater. This enzyme demonstrates polyextremophilic properties, functioning effectively in high temperatures, high salt concentration, and acidic pH.

In aquaculture, which also produces saline wastewater, Anh et al. (2021 B) suggested using halophilic microorganisms and their enzymes in wastewater treatment. They specifically emphasize the importance of proteases in this process. According to their review, moderately halophilic organisms, which can tolerate NaCl concentrations below 10 g $L^{-1}$, are more effective due to their adaptability to varying salt levels. Anh et al. (2021 A) also proposed coculturing as a method to enhance proteolytic activity. They found that proteolytic activity was 1.8–2.4 times higher in cocultures compared to pure cultures and remained active across different environmental conditions (temperatures from 25 °C to 60 °C, pH 4–12, and salinity levels of 100–300 g $L^{-1}$). The authors conducted these experiments using *Marinirhabdus sp.* and *Marinobacter hydrocarbonoclasticus*, which were isolated from Port Philip Bay seawater, Australia (Table2).

The use of not only halophilic enzymes but also microorganisms themselves can be effective in wastewater treatment. According to Tan *et al.* (2019), using such microorganisms in wastewater treatment can eliminate the need for its previous desalination before entering the biological steps. The study also emphasizes the importance of understanding the metabolic processes of halophiles across different levels of salinity. Furthermore, research on the use of halophiles in a single-chambered air cathode microbial fuel cell (ACMFC) for wastewater treatment and energy production indicated that employing halophiles can help address the salinity issue in these processes (Jamal *et al.* 2020).

## BIOREMEDIATION

Hydrocarbon contamination is hazardous for all living organisms, including humans. Due to the extensive use of petroleum as an energy source and as raw material for various products, accidents involving petroleum spills occurs relatively frequently. One eco-friendly approach to dealing with environmental contamination from oil involves the use of microorganisms to degrade hydrocarbons, a process called bioremediation (Ghaedrahmat *et al.* 2022). However, under certain circumstances (e.g., soil contamination by oil and saline produced water resulting from pipeline failure), high salinity may reduce the rate of hydrocarbon biodegradation. This occurs due to the detrimental effect of excess salt on mesophilic hydrocarbon-degrading microorganisms, which do not withstand high salt concentrations. In such situations, salt-tolerant microorganisms become an option for hydrocarbon bioremediation (Pourfadakari *et al.* 2021).

Halotolerant microorganisms that can degrade hydrocarbons are more effective when they produce biosurfactants. Biosurfactants increase the availability of hydrocarbons by reducing the interfacial tension between these hydrophobic molecules and water. Additionally, biosurfactant-producing bacteria can be used in Microbial Enhanced Oil Recovery (MEOR) (Somee et al. 2021). In this context, our research group screened halotolerant bacteria from Trindade Island, ES, Brazil that could produce biosurfactants that remained active up to 175 g $L^{-1}$ NaCl. The study concluded that these bacteria may be useful for bioremediation and oil recovery in saline environments. Additionally, in an unpublished study, our group used halotolerant strains of *Virgibacillus sp.*, *Bacillus glycinifermentans*, and *Bacillus licheniformis*, also isolated from Trindade Island, to form a consortium. This consortium demonstrated high efficiency in degrading hydrocarbons in soils contaminated with crude oil and hypersaline wastewater through biostimulation using the compatible solute glycine betaine (da Silva *et al.* 2015).

In 2019, Ibrahim *et al.* conducted a study on exopolysaccharides with biosurfactant properties from *Halobacillus sp.* isolated from a salt sample from Lake Qarun, Egypt. They suggested that this bacterium could be used for bioremediation purposes. Another study found that not only halophilic Bacteria, but also Archaea, can be effective in bioremediation. The authors isolated five alkane-degrading halophilic archaeal strains from a hypersaline site in Camargue, France, which had historical hydrocarbon contamination. The results showed positive effects of the archaeal strains in the biodegradation of hydrocarbons in hypersaline environments (Tapilatu *et al.* 2010).

A recent study with halotolerant yeasts described the ability of these microorganisms to form biofilms, which increases their resilience to environmental stressors. This characteristic may be favorable for their survival in contaminated saline environments and increase their efficacy in bioremediation efforts. Moreover, their exopolysaccharides exhibit biosurfactant properties, further facilitating the biodegradation of hydrophobic contaminants (Andreu & Del Olmo, 2023). The halophilic filamentous fungi *Aspergillus sydowii,* isolated from sugarcane bagasse, is capable of consuming polycyclic aromatic hydrocarbons (PAH) in a hypersaline medium. When growing on PAH, its genes related to starvation, cell wall remodeling, degradation and metabolism of hydrocarbons, DNA/RNA metabolism, energy generation, signaling, and general stress responses are upregulated (Peidro-Guzmán *et al.* 2021) (Table2).

**Table 2** Halotolerant/Halophilic microorganisms with applications in wastewater treatment and bioremediation.

| **Halotolerant/Halophilic microorganism** | **Domain** | **Site** | **Application** | **Reference** |
|---|---|---|---|---|
| *Bacillus cereus* | Bacteria | Soak Liquor | Production of halotolerant lipase that remove fat from saline wastewater from the leather industry | Pounsamy et al. (2019) |
| *Aspergillus gracilis* | Eukarya | Man-made saltern in Thailand | Production of amylase that have higher activity across various salt concentrations in simulated wastewater | Ali *et al*. (2014) |
| *Marinirhabdus sp.* and *Marinobacter hydrocarbonoclasticus* | Bacteria | Port Philip Bay seawater, Australia. | High proteolytic activity when cocultured in medium containing salinity levels of 100–300 g $L^{-1}$ | Anh *et al*. (2021 A) |
| *Halobacillus sp.* | Bacteria | Lake Qarun, Fayoum Province, Egypt | Production of exopolysaccharides with biosurfactant properties | Ibrahim *et al.* (2019) |
| *Virgibacillus sp.*, *Bacillus glycinifermentans*, and *Bacillus licheniformis*, | Bacteria | Trindade Island, Brazil | Degradation of hydrocarbons in soils contaminated with crude oil and hypersaline wastewater through biostimulation using the compatible solute glycine betaine | Unpublished data |
| *Providencia rettgeri*, *Proteus mirabilis, Pseudomonas aeruginosa*, and *Enterococcus faecalis* | Bacteria | Saline tannery wastewater | Wastewater treatment | Maharaja *et al.* (2017);<br>Maharaja *et al.* (2018);<br>Maharaja *et al.* (2019);<br>Sivaprakasam *et al.* ( 2011) |

## HALOPHILES IN ASTROBIOLOGY

Beyond industry, halophilic and halotolerant microorganisms have significant implications for astrobiology, which is the study of the origin, evolution, distribution, and future of life in the universe. Although human curiosity about extraterrestrial life can be traced back thousands of years, the term 'astrobiology' was only coined in 1998 as a replacement for 'exobiology', which did not include life on Earth. Astrobiology is based on the study of life on our planet, the life that we know, and is concerned with how life may behave outside the Earth while also contributing to our understanding of life on Earth, encompassing various research areas (Blumberg 2003; Dick 2012; Yamagishi 2019).

Among these research areas, we will focus on the study of microorganisms from extreme environments. These extreme environments are widespread on Earth and serve as analogs of environments found on other worlds, like Mars, Jupiter's moon Europa, and Saturn's moon Enceladus, which are of prime importance to astrobiological studies (McKay 2008; Preston & Dartnell 2014). High salt concentration sites are likely present on these three celestial bodies (Ashkenazy & Tziperman 2021; Cockell 2014; Zolotov 2007), and organisms that can tolerate high salt concentrations could serve as possible models for studying life on these worlds.

The study of extremophiles as model organisms for astrobiology is a recent development. As a result, only a few organisms have been reported so far (Emlik & Marakli, 2023). Among them are bacteria such as *Colwellia psychrerythrae* and *Planococcus halocryophilus*, which can survive in salty and cold environments. Additionally, the Antarctic archaea *Halorubrum lacusprofundi* can grow in perchlorate salts that are possibly abundant on Mars (Laye & DasSarma 2018; Mudge *et al.* 2021; Waajen *et al.* 2020). Another halophilic archaea, *Halobacterium salinarum* NRC-1, can withstand not only high salt concentrations but also high UV radiation and high and low temperatures. Both archaea were able of survive in stratospheric conditions inside helium balloons, but *H. lacusprofundi adapted better*, likely because of its higher tolerance to low temperatures (DasSarma *et al.* 2017). Moreover, haloarchaea have been studied for their ability to produce pigments that can be detected by remote sensing. With future technologies, they may be detected on planets and moons located in habitable zones (DasSarma *et al.* 2020).

*Deinococcus radiodurans*, a bacterium that remains metabolically active under high UV irradiation, has also been studied as an astrobiological model organism. It was capable of surviving three years outside the International Space Station (ISS) in a protected compartment during a study (Kawaguchi *et al.* 2013; Venkateswara *et al.* 2000). Furthermore, cyanobacteria of the genus *Chroococcidiopsis sp.* can survive ionizing radiation, desiccation, and oxidative stress, in addition to high UV irradiation (Li *et al.* 2022; Napoli *et al.* 2021; Verseux *et al.* 2017).

Among eukaryotes, fungi such as *Cladosporium sphaerospermum* and *Aspergillus fumigatus*, isolated from Chernobyl and the ISS Hepa filter, respectively, have withstood simulated Mars conditions (Blachowicz *et al.* 2019). The yeast *Debaryomyces hansenii* has been studied for its resistance to sodium perchlorate (Heinz *et al.* 2020) (Table 3). Additionally, black yeasts are poliextremophilic models that survive Martian analog conditions (Schultz *et al.* 2023).

Most of the cited model organisms are reported as salt-tolerant (Gostinčar & Gunde-Cimerman 2024; Mansour 2017; Navarrete *et al.* 2021), probably because saline environments are widespread in our solar system (Bryanskaya *et al.* 2013). In this context, the following sections will delve into the application of halophiles and halotolerants in astrobiology studies, with a particular emphasis on Mars, Europa, and Enceladus.

## MARS

The search for extraterrestrial life is closely related to the presence of water. Although Mars' surface currently lacks the conditions to host liquid water due to low pressure and temperature (O'Callaghan 2020; Qin *et al.* 2023; Wray 2021), this was not always the case. Researchers have reported the presence of liquid water during the Noachian period (4.1-3.7 Ga), in the form of oceans, rivers and lakes that had vanished by the Hesperian (3.7-3.0 Ga) (Andrews-Hanna and Lewis 2011; Head *et al.* 1999; Kite 2019; Selese *et al.*2021). The Noachian period also had evidence of geologic activity, with volcanic deposition, magma precipitation, and hydrothermal activity (Meunier *et al.* 2012; Quantin *et al.* 2012).

Despite the currently harsh conditions on the surface, the ice-rich Martian subsurface may contain liquid water protected from freezing by lithostatic pressure, radiogenic heating, and salts, especially perchlorate (Hanna & Phillips 2005; Morgan *et al.* 2021; Nuding *et al.* 2014; Ojha *et al.* 2021). Perchlorate salt has been found on Mars by Phoenix, in a concentration of 0.4-0.6 wt %, and can maintain water in the liquid state due to its deliquescence property (Chevrier *et al.* 2022; Kounaves *et al.* 2014). The subsurface could protect current or past life from UV radiation (fig.2), and the planet can offer the main elements for life, including carbon, hydrogen, oxygen, nitrogen, phosphorus, sulfur, and trace elements (House *et al.* 2022; Hu & Thomas 2022; Joseph *et al.* 2020; Mitrofanov *et al.* 2022; Moreras-Marti *et al.* 2022; Payre *et al.* 2017; VanBommel *et al.* 2023; Viudez-Moreira 2021).

The subsurface microbiome on Earth is believed to be just as rich as the one on the surface. Additionally, the evaporation of oceans and saline lakes has enabled the formation of brines, which can harbor halophilic microorganisms (Stan-Lotter 2019). Halophiles can survive, by definition, to high concentrations of NaCl, but some studies have shown that they can also thrive in the presence of other salts. For example, the haloarchaea *Haloferax mediterranei*, *Haloferax denitrificans*, *Haloferax gibbonsii*, *Haloarcula marismortui*, and *Haloarcula vallismortis* were able to grow at ~ 49 g $L^{-1}$ of sodium perchlorate and used perchlorate as an electron acceptor in the absence of oxygen, despite the toxicity of this salt for other organisms (Oren *et al*. 2014).

Perchlorate is thought to be toxic due to its high oxidative capacity, but halophiles have demonstrated high resistance to oxidative stress (Laye and DasSarma 2018). Another example of perchlorate tolerance by halophiles is found in bacteria from Big Soda Lake, Nevada, USA, which were capable of growth up to 20 g $L^{-1}$ perchlorate (Matsubara *et al.* 2017). *Planococcus halocryophilus* Or1, a non-spore forming aerobic heterotrophic bacterium isolated from active layer soil from Eureka (Ellesmere Island, Nunavut, Canada) (Mykytczuk *et al*. 2012), is able to grow from -15 $^{o}$C to 37 $^{o}$C and withstand NaCl concentration up to 180 g $L^{-1}$. It tolerates high concentrations of sodium, magnesium, and calcium perchlorate and chloride, increasing its tolerance when transferred from a lower salt concentration to a higher one sequentially (1 wt% [w/w] incremental steps) (Heinz *et al.* 2019).

The possible habitable environments on Mars have implications for planetary protection. Even though no life has been found on Mars so far, the habitable features of the planet make contamination with Earth microorganisms a concern during spacecraft exploration (Changela *et al.* 2021). While spacecraft assembly cleanrooms on Earth go through rigorous decontamination processes, microorganisms have still been found (Hendrickson *et al.* 2021; Maltais *et al.* 2020). To address this, Mettler *et al.* (2023) developed Martian saline seep models to investigate the possible establishment of biofilms from a terrestrial saline seep in Montana (USA). The study found the genera *Halomonas*, *Planococcus*, and *Bacillus*, which were also detected in the cleanrooms. This study shows that microorganisms of the same genera found in the spacecraft assembly cleanrooms can survive in Martian analog conditions. This is a significant concern due to ability of these microorganisms to form biofilms, which protects microorganisms from environmental stress and antimicrobials (Table 3).

## ICY MOONS

Europa and Enceladus are two moons in our solar system that are believed to have oceans covered by ice (Kang 2022). It is expected that these oceans are salty and are kept in a liquid state due to tidal heating and hydrothermal vents (Deamer & Damer 2017; Kang *et al.* 2022; Matsuyama *et al.* 2018). On Earth, hydrothermal vents are hotspots of life, and one of the theories of the origin of life relies on the hypothesis that life originated in hydrothermal vents. These sites offer energy sources and an environment propitious to chemical reactions (Martin *et al.* 2008). Therefore, Europa and Enceladus not only have liquid water but may also provide environments where life could originate and evolve.

Europa is one of Jupiter's Galilean moons. It has a magnetic field and a young surface that, combined with tidal flexing and a subsurface ocean, suggests current geological activity (Phillips and Pappalardo, 2014). Based on data from the Galileo mission, the salt content of Europa's ocean is estimated to be higher than 50 practical salinity unit (psu) (Hand & Chyba 2007; Kang *et al.*, 2022). The ocean's composition is still uncertain, since the recent confirmation of NaCl on Europa has led scientists to revise the previous notion of an ocean dominated by sulfate salts, with the possibility of a carbonate ocean even being suggested (Melwani *et al.* 2021; Trumbo *et al.*, 2022).

Enceladus, one of Saturn's moons, is believed to have active geological processes due to the plume that emerges from it. This plume is made up of vapor and particles. The vapor consists mainly of water vapor, with lower concentrations of $CO_2$, $NH_3$, $CH_4$, and organic molecules. The particles, on the other hand, are mostly composed of water and ice, but salts such as NaCl have also been detected (Spencer & Nimmo 2013). Additionally, molecular hydrogen has been found in the plume, indicating the presence of hydrothermal activity on the ocean floor (Waite *et al.* 2017).

How the heat from Enceladus hydrothermal vents is distributed throughout the ocean is closely related to the salinity of the water. Even though Cassini measured a salinity of 5 to 20 psu, the actual salt concentration remains unknown. This is because the salt concentration is likely to be higher than that indicated by the samples collected from the south pole sprays, which lost salinity through condensation (Kang *et al.*, 2022; Postberg *et al.*, 2009). The dominant salts of the ocean of Enceladus are NaCl and $NaHCO_3$ or $Na_2CO_3$, as suggested by analysis of solid ice grains (Fifer *et al.* 2022).

Ancient halophilic microorganisms have been found preserved in salt deposits on Earth, which suggests that the same could happen on other worlds. Halite, a mineral that is abundant throughout the Solar System, including on Mars, Europa and Enceladus, could potentially preserve microorganisms as well. Europa and Enceladus have salty oceans and hydrothermal heat, which makes it plausible to consider the existence of halophiles on these moons (Bryanskaya *et al.* 2013; Stan-Lotter *et al.* 2012). Weber *et al.* (2023) have highlighted the importance of the salt concentration and composition of these oceans in theorizing biotic processes that could occur on these icy moons. The authors suggest that the most likely metabolism on Enceladus is methanogenesis, while on Europa it is methane oxidation and sulfate reduction.

**Table 3** Halotolerant/Halophilic microorganisms applicable to astrobiology studies

| Halotolerant/Halophilic microorganism | Domain | Site | Application | Reference |
|---|---|---|---|---|
| *Colwellia psychrerythrae* | Bacteria | Subzero Arctic marine sediments | Maintain activity in subzero temperatures and high salinities. Search for life in Icy worlds. | Mudge *et al.* (2021) |
| *Halorubrum lacusprofundi* | Archaea | Deep Lake in the Vestfold Hills, Antarctica | Withstanding perchlorate salts and stratospheric conditions; Survival in Martian analog conditions. | Franzmann e*t al*. (1988); Laye & DasSarma (2018) |
| *Halobacterium salinarum* NRC-1 | Archaea | NR | Withstanding high salt concentrations, high UV radiation, high and low temperatures, and stratospheric conditions. | DasSarma *et al.* (2017) |
| *Planococcus halocryophilus* Or1 | Bacteria | Active layer soil from Eureka (Ellesmere Island, Nunavut, Canada) | It is able to grow from -15 ºC to 37 ºC, and tolerates high concentrations of sodium, magnesium, and calcium perchlorate and chloride | Mykytczuk *et al.* (2012); Heinz *et al.* (2019) |
| *Cladosporium sphaerospermum* | Eukarya | Chernobyl | Survival in Martian analog conditions. | Blachowicz *et al.* (2019) |
| *Haloferax mediterranei*, *Haloferax denitrificans*, *Haloferax gibbonsii*, *Haloarcula marismortui*, and *Haloarcula vallismortis* | Archaea | NR | They were able to grow at ~ 49 g $L^{-1}$ of sodium perchlorate and used perchlorate as an electron acceptor in the absence of oxygen | Oren & Mana, (2014) |

## CONCLUSION

Halophilic and halotolerant microorganisms hold significant promise for applications in both industry and astrobiology. Despite being underexplored in industry, these organisms have demonstrated their potential in producing industrial enzymes and have been studied for application in saline wastewater treatment plants and bioremediation of saline environments, among other applications. Their environmentally friendly and economically advantageous nature makes them suitable substitutes for non-biological treatment processes and for other organisms conventionally used in wastewater treatment and bioremediation that can not withstand high salt concentrations. In the realm of astrobiology, halotolerant or halophilic microorganisms serve as models for other possibly habitable worlds beyond Earth, and the environments where they thrive are considered analogs to those on other planets and moons. Their ability to survive in the presence of various salts, including those expected to be found on Mars, Europe and Enceladus, underscores their potential significance in the search for life beyond Earth. Additionally, the polyextremophile character of these organisms makes them able to thrive in other extraterrestrial extreme conditions such as under high UV incidence, low temperatures from Mars and Icy moons, and high temperatures from hydrothermal vents from Europa and Enceladus and volcanoes of ancient Mars.

The importance of studying the adaptive mechanisms of halophiles and halotolerants cannot be overstated, as it has implications for both biotechnological and astrobiological applications. By understanding these mechanisms at the physiological and molecular levels, we can better identify industrially and astrobiologically applicable organisms using metagenomics and explore possibilities for improving tolerance capabilities through genome manipulation. Genomic, transcriptomic, and proteomic studies of halophiles and halotolerants are therefore crucial in advancing our understanding of these organisms and their potential for application in various fields. In light of these considerations, we propose a comprehensive study that integrates laboratory adaptive evolution and “omics” studies to delve into the adaptive mechanisms of halophiles and halotolerants. Such research will not only contribute to our understanding of how these organisms have evolved on Earth but also shed light on their potential for adaptation and evolution in other worlds, thereby furthering our exploration of life beyond our planet.

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# CHAPTER II: ASTROBIOLOGICAL AND BIOTECHNOLOGICAL POTENTIAL OF HALOTOLERANT BACTERIA ISOLATED FROM BRINE, MANGROVE, DUNES, AND BEACH SOILS.

## INTRODUCTION

The study of extremophiles has gained significant attention in recent years due to their diverse scientific and biotechnological applications, which result from their remarkable adaptations to harsh environments. Extremophiles can be classified into various categories based on the extreme conditions they are capable of withstanding. These categories include thermophiles, hyperthermophiles, psychrophiles, psychrotolerants, acidophiles, alkaliphiles, barophiles, halophiles, and halotolerants (Ali et al., 2023).

Halotolerant bacteria are different from halophiles in that they do not require NaCl to live, but can withstand high concentrations of this salt (Larsen 1986). Non halophiles/non halotolerants are affected by high NaCl concentrations, losing cellular water by osmosis, but salt-tolerant microorganisms have adaptive mechanisms that allow them to survive under these extreme conditions (Mukhtar et al. 2020). The salt-in mechanism maintains the ionic concentration in the cell through ions symport, antiport, and active transport (Gunde-Cimerman et al. 2018). In contrast, the salt-out mechanism maintains the osmotic pressure through the synthesis or uptake of compatible solutes such as glycine betaine and trehalose (Ionescu et al. 2024).

Another characteristic adaptation of halophiles is the higher number of acidic amino acids in their enzymes, compared to non-halophiles (Madern et al. 1995). These adapted enzymes have applications in various industry sectors. They can maintain activity not only at high salt concentrations but also at extreme pH levels and temperatures (Delgado-García et al. 2012). Halophilic enzymes can be used to treat saline wastewater. Lipases, amylases, and proteases have been studied for treating wastewater from the leather industry. This type of wastewater is rich in NaCl and mainly consists of lipids, mucopolysaccharides, and proteins (Maharaja et al. 2020). Enzymatic wastewater treatment is more effective than chemical treatment due to faster reaction kinetics and lower water and energy consumption (Feng et al. 2021). Additionally, not only immobilized enzymes can be applied, but also the bacteria itself using bioreactors with a microbial consortium. The use of salt-tolerant bacteria can replace the conventional biological treatment, which may require an expensive and laborious desalination step (Tan et al. 2019).

Halophilic microorganisms are also used for the bioremediation of saline-contaminated aquatic and terrestrial environments. Oil contamination poses a risk to all forms of life, including humans. In this context, microorganisms can be used to degrade contaminants. This process, besides being effective, is simple, economical, and does not cause significant environmental damage (Ghaedrahmat et al. 2022). However, most organisms currently used for this purpose do not withstand or degrade contaminants in high salt concentrations, highlighting the importance of studies on halophiles for bioremediation (Zhao et al. 2023).

Besides industrial and environmental applications, salt-tolerant organisms are models for astrobiological studies. Mars contains elements necessary for the origin and maintenance of life, and there are evidence of the presence of liquid water on that planet. However, the presence of perchlorate salts on the surface and in subsurface brines may pose a challenge to life, since these compounds my damage microbial cells by inducing oxidative stress and biomolecules destabilization (Cockel, 2014; Heinz *et al.* 2021). Despite this, halophilic organisms have shown to resist to oxidative stress caused by perchlorate (Oren *et al.* 2013; Laye and DasSarma 2018). Some halophilic organisms have demonstrated the ability to survive at perchlorate concentrations higher than those found on Mars (0.4-0.6 wt %) (Flores *et al.* 2020; García-Descalzo *et al.* 2023). On icy moons like Enceladus and Europa, NaCl is the main salt present in varying concentrations in their saline oceans (Ashkenazy and Tziperman 2021; Trumbo *et al.* 2022; Weber *et al.* 2023). Both moons contain important elements for life and are geologically active (Kang et al., 2022; Weber et al., 2023).

Halotolerant bacteria can be found in various places in Brazil, such as the mangroves, Araruama Lagoon in Rio de Janeiro, and the brines in Ceará and Rio Grande do Norte (Camara 2021; Cardoso et al. 2011; Duarte et al. 2012). Ceará state has historical significance in Brazil's salt economy. It attracted the Dutch garrison during the colonial period due to the presence of natural salt flats in the municipality of Camocim (Diniz and Vasconcelos, 2016). Although Brazil hosts numerous saline environments and salt-tolerant microorganisms hold significant potential for economic, environmental, and astrobiological applications, the Brazilian halotolerant microbiome remains largely underexplored.

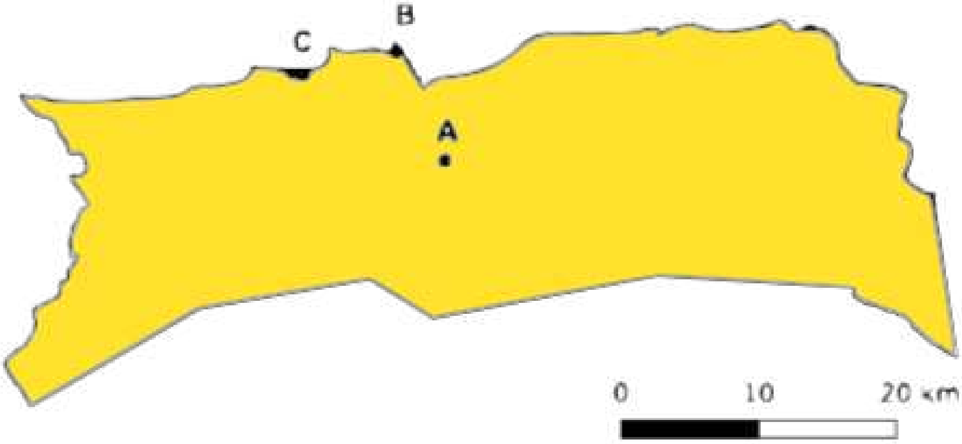
This study aimed to investigate halotolerant bacteria isolated from different areas of Camocim, Ceará, Brazil. We evaluated the environmental and industrial applications of the strains by analyzing their capacity to produce biosurfactants, grow using hydrocarbons as the sole carbon source, produce exopolysaccharides and the enzymes lipase, protease, and amylase. Additionally, we explored heir potential application in astrobiology studies by studying their tolerance to sodium perchlorate and the ability to use this salt as the sole electron acceptor.

## MATERIALS AND METHODS

### Isolation and maintenance of bacterial strains

Strains were isolated from soils collected in Camocim, Ceará, Brazil in three distinct areas. These areas include the brine owned by the company Umari Salineira, at bare and rhizospheric soil, and the rhizospheric soil of the mangroove (2°55'59"S 40°49'50"W). Additionally, rhizospheric soil of herbaceous plants at Praia do Farol (2°51'43"S 40°51'44"W), and bare (2°52'33"S 40°55'48"W) and rhizospheric (2°52'36"S 40°55'24"W) soils of the dunes at Estrada para Maceió were also included  (Fig. 1). The bare soil samples were collected at a depth of 0 to 5 centimeters. The isolation of halophilic and halotolerant bacteria was performed on a Tryptone Soy Agar (TSA) medium containing 100 g $L^{-1}$ and 50 g $L^{-1}$ NaCl, at 30 °C and 50 °C. The microorganisms were preserved by cryopreservation using glycerol at -80 °C. The microorganisms were reactivated in Tryptic Soy Agar (TSA) and subsequently inoculated into Tryptic Soy Broth (TSB), both media prepared without the addition of NaCl prior to the subsequent experiments

**Fig. 1** Map of the collection areas. Collections sites are represented with red dots. A = brine area (2°55'59"S 40°49'50"W), B = Praia do Farol (2°51'43"S 40°51'44"W), C = Dunes (bare: 2°52'33"S 40°55'48"W and rhizospheric: 2°52'36"S 40°55'24"W).



## Identification

Strain identification was initially performed using the fatty acid methyl ester (FAME) analysis protocol recommended by MIDI Inc., based in Newark, Delaware, USA. FAMEs were analyzed using MIDI Sherlock® software with the TSB method and library. The organisms were further identified by 16S rRNA sequencing. The extraction of total DNA was carried out using the Biospin Bacteria Genomic DNA Extraction Kit by BioFlux according to the manufacturer's instructions. The amplification of the 16S rRNA coding sequence was done using universal primers for bacteria 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The thermal cycling conditions included an initial denaturation at 95°C for 2 minutes, followed by 34 cycles of denaturation at 95°C for 2 minutes, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, with a final extension at 72°C for 5 minutes. Amplifications were carried out in a thermal cycler. The products resulting from the Polymerase Chain Reaction (PCR) were evaluated by agarose gel electrophoresis, with quantification done using Qubit® The PCR products were purified using the Ultraclean PCR purification kit according to the manufacturer's instructions and sequenced by Sanger method using an ABI sequencer. The nucleotide sequence identities were compared to those available in the National Center for Biotechnology Information (NCBI) database. A phylogenetic analysis of the organisms was performed using the Molecular Evolutionary Genetics Analysis (MEGA X) software. The evolutionary history was inferred using the Maximum Likelihood method and the Kimura 2-parameter model, with bootstrap analysis based on 10,000 replicates to assess the robustness of the phylogenetic tree.

## Sodium chloride and perchlorate tolerance

Tolerance experiments to sodium chloride and perchlorate were conducted in 96-well microplates. The microorganisms were grown at 30°C in Tryptic Soy Broth (TSB) supplemented with 100, 150, and 200 g $L^{-1}$ NaCl, or with 10, 30, 50, 70, 90, and 110 g $L^{-1}$ $NaClO_4$. The incubation was carried out in a BioTek microplate reader at 30°C for 72 hours, with growth measured by Optical Density at 600 nanometers ($OD_{600}$) every 30 minutes. Data were stored and analyzed using the Gen5 software. The evaluation of salt tolerance was performed by calculating the specific growth rate ($\mu$) using the formula $\mu = \ln_2/G$, where $G$ represents the generation time. We compared the salt tolerance among the strains using ANOVA (Analysis of Variance) and Tukey's post-hoc test for the concentrations for the data that met ANOVA assumptions and the Kruskal-Wallis test followed by Dunn's post-hoc test for those that did not. Statistical analyses were made using the R software.

## Perchlorate reduction

The ability of microorganisms to grow in an anoxic environment using perchlorate as the sole electron acceptor was analyzed by transferring 1 mL of a culture obtained in Anaerobic Lactate-Perchlorate Medium (ALP) to 15 mL penicillin vials containing Low-Mineral Medium (LMM), which was supplemented with 10 mM NaClO4 and purged with nitrogen ($N_2$) to eliminate oxygen ($O_2$) (Bruce et al. 1999; Wang et al. 2018). A control group was established by inoculating microorganisms into anaerobic LMM medium without perchlorate. After 14 days of incubation, bacterial growth was assessed by measuring dry biomass in the medium and by visual assessment using optical microscopy/simple staining. To assess cellular viability, we conducted an ATP assay utilizing a luciferase probe (Ludin, 2000). The culture was prepared in the previously mentioned LMM medium in a 96-well microplate, following incubation in an anaerobic chamber at 35°C. After 14-day, we used BacTiter-GloTM Microbial Cell Viability Assay to measure luciferase luminescence using a Thermo Scientific Varioskan Lux.

## Biosurfactant production and growth in hydrocarbon medium

All microorganisms were tested for their ability to produce biosurfactants using the drop collapse technique described by A. Bodour and Miller-Maier (1998). Briefly, the inoculum was prepared in TSB medium and then incubated at 30°C and 200 rpm for 24 or 48 hours, depending on the bacterium growth rate. Bacteria that showed positive results in the initial test had the surface tension of the growth medium measured using a Dataphysics tensiometer and the DCATS 31 software to confirm the reduction in surface tension. To assess the growth of the strains using hydrocarbons as the sole carbon source, the strains were grown in MSM medium with 2% (v/v) hexadecane for 15 days. After this time, resazurin was added as an activity indicator (active cells reduce resazurin to resorufin, a pink compound).

## Synthesis of exopolysaccharides

We assessed synthesis of exopolysaccharide by streaking the strains on Luria-Bertani Agar (LBA) containing 0.2 g $L^{-1}$ Calcofluor. Fluorescent colonies under UV-C light exposure were considered positive for exopolysaccharide production (Leigh et al., 1985).

## Enzymatic production

Enzyme production was assessed in 0.1X LBA medium with 10 g $L^{-1}$temperature and at 50°C for the others, at 200 rpm for 24 or 48 hours, depending on the strain. The $OD_{600}$ were determined and normalized to 0.1, and 10 µL of the culture was spotted onto plates containing the enzyme substrates. The plates were then incubated at 30°C or 50°C. The presence of the enzymes was determined by the appearance of clear zones around the culture.

# RESULTS AND DISCUSSION

## Identification

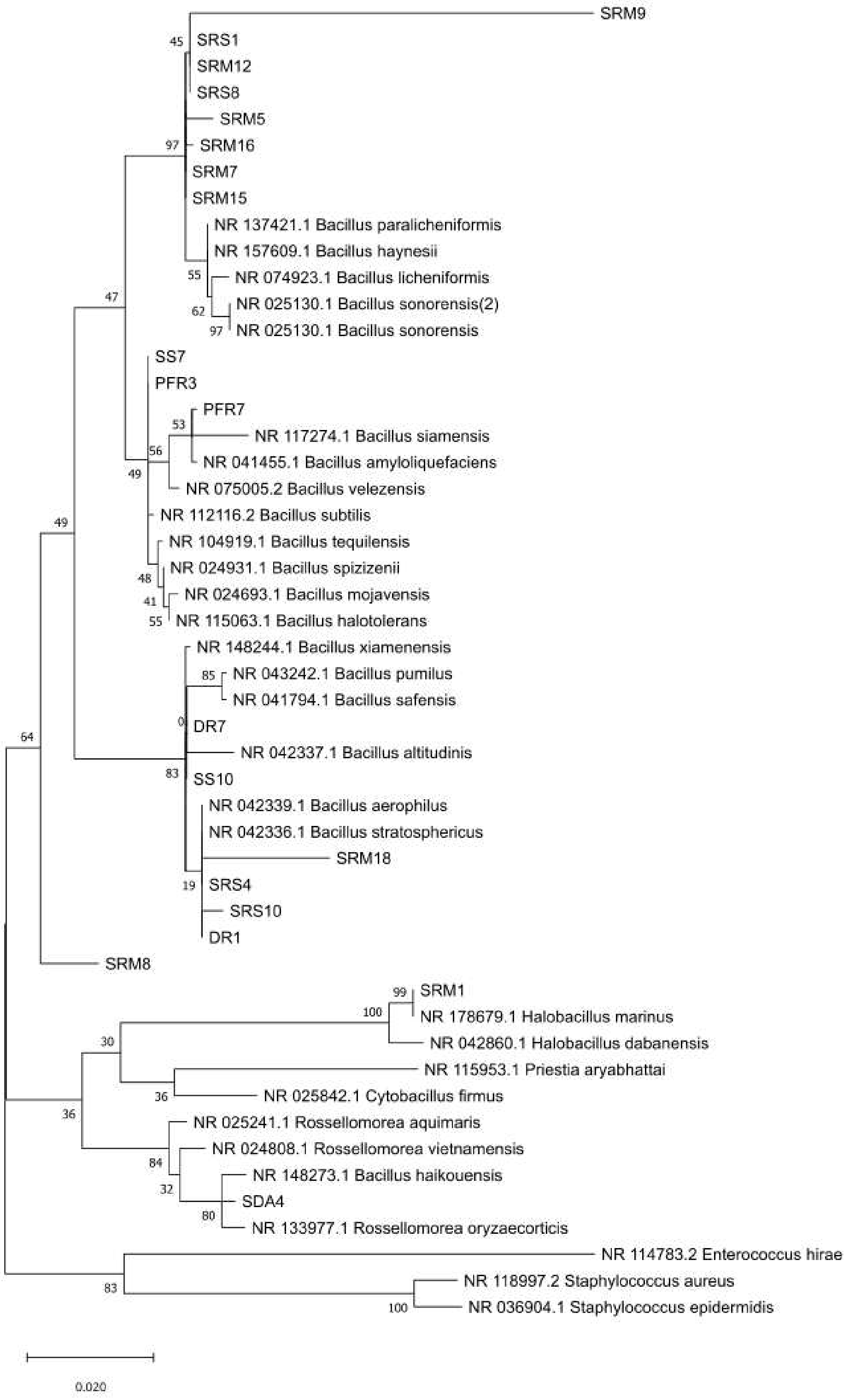
Fatty acids analysis did not provide clear identification at the species level, except for CaSRM9, identified as *Bacillus licheniformis* (SIM index > 0.8). The SIM index was between 0.6 and 0.8 for five strains identified as *Bacillus*, which agreed with the phylogenetic analysis using rRNA 16s. The phylogenetic analysis suggests that most strains belong to the *Bacillus* genus. CaSDA4 showed higher similarity to the *Rossellomorea* genus, especially *Rossellomorea vietnamensis,* and CaSRM1 to *Halobacillus* genus, especially *Halobacillus marinus,* which are part of the *Bacillus* clade (Gupta *et al*. 2020; Spring *et al*. 1996). The predominance of *Bacillus* among strains isolated from saline medium aligns with previous studies that frequently report halotolerant *Bacillus* worldwide (Nas *et al*. 2021; Nimkande *et al*. 2023; Orhan and Gulluce 2015; Reang *et al*. 2024). Table 1 presents the strains along with their classification based on fatty acid composition and 16S rRNA sequencing, as well as their respective isolation sites. Fig. 1 illustrates the phylogenetic relationships among the strains and closely related species.

**Table 1** Identification of Strains Using FAME (MIDI Sherlock) and 16S rRNA, Including Isolation Sites and Similarity Indices

*A similarity index (SIM index) < 0.6 indicates low similarity, 0.6-0.8 indicates moderate similarity, and >0.8 indicates high similarity. Five strains did not match any entries in the MIDI Sherlock TSB library.

| Strain | Site | ID FAME (SIM index) | ID 16s |
|---|---|---|---|
| CaSDR1 | Dune rhizospheric soil | *Bacillus megaterium* (0.593) | *Bacillus sp.* |
| CaSDR7 | Dune rhizospheric soil | *Pseudomonas oryzihabitans (*0.282) | *Bacillus sp.* |
| CaSDA4 | Dune bare soil | *Brevibacillus formosus* (0.319) | *Rossellomorea sp.* |
| CaPFR3 | Praia do Farol rhizospheric soil | *Bacillus subtilis* (0.693) | *Bacillus sp.* |
| CaPFR7 | Praia do Farol rhizospheric soil | *Paenibacillus macerans* (0.414) | *Bacillus sp.* |
| CaSRS1 | Brine rhizospheric soil | - | *Bacillus sp.* |
| CaSRS4 | Brine rhizospheric soil | *Bacillus alcalophilus* (0.366) | *Bacillus sp.* |
| CaSRS8 | Brine rhizospheric soil | - | *Bacillus sp.* |
| CaSRS10 | Brine rhizospheric soil | *Bacillus pumilus* (0.642) | *Bacillus sp.* |
| CaSS7 | Brine bare soil | - | *Bacillus sp.* |
| CaSS10 | Brine bare soil | *Bacillus pumilus* (0.621) | *Bacillus sp.* |
| CaSRM1 | Mangrove rhizospheric soil | *Bacillus atrophaeus* (0.696) | *Halobacillus sp.* |
| CaSRM5 | Mangrove rhizospheric soil | *Bacillus sp.* (0.209) | *Bacillus sp.* |
| CaSRM7 | Mangrove rhizospheric soil | - | *Bacillus sp.* |
| CaSRM8 | Mangrove rhizospheric soil | *Bacillus sp.* (0.406) | *Bacillus sp.* |
| CaSRM9 | Mangrove rhizospheric soil | *Bacillus licheniformis* (0.812) | *Bacillus sp.* |
| CaSRM12 | Mangrove rhizospheric soil | - | *Bacillus sp.* |
| CaSRM15 | Mangrove rhizospheric soil | *Microbacterium barkeri* (0.575) | *Bacillus sp.* |
| CaSRM16 | Mangrove rhizospheric soil | *Bacillus subtilis* (0.627) | *Bacillus sp.* |
| CaSRM18 | Mangrove rhizospheric soil | *Halomonas aquamarina* (0.425) | *Bacillus sp.* |

**Fig.2** Phylogenetic tree illustrating the relationships among the strains and closely related species.



### Sodium chloride tolerance

All 20 tested strains were able to withstand 100 g $L^{-1}$ NaCl, with CaSRM5 showing the highest mean specific growth rate at this salt concentration ($\mu = 0.326$ $h^{-1}$). Eleven strains were able to grow at 150 g $L^{-1}$, with CaSDR7 reaching the highest specific growth rate at this salt concentration ($\mu = 0.241$ $h^{-1}$). This strain did not perform as well at the lower salt concentration ($\mu = 0.125$ $h^{-1}$). Only CaSS7 was able to grow at 200 g $L^{-1}$, albeit very slowly ($\mu = 0.0164$ $h^{-1}$). Despite being able to withstand higher concentrations of NaCl, CaSS7 presented lower $\mu$ values at lower salt concentrations compared to the other strains ($\mu = 0.060$ $h^{-1}$ at 100 g $L^{-1}$ and 0.032 at 150 g $L^{-1}$). These results indicate no direct relation between the growth rate in the presence of NaCl and tolerance to higher concentrations of this salt. ANOVA assumptions were not met by the $\mu$ values at 100 and 150 g $L^{-1}$. Therefore, we analyzed the data using the Kruskal-Wallis test and Dunn test, which showed significant differences among the strains. Using the suggested classification of Larsen (1986), the strains obtained in this study were classified as moderate halotolerant (organisms which withstand up to 180 or 200 g $L^{-1}$ NaCl) (Tables 2 and 3).

**Table 2 -** Specific growth rates (μ) of the bacterial strains at 100 g $L^{-1}$ and 150 g $L^{-1}$ NaCl. Strains labeled with different letters indicate significant differences of their respective specific growth rates at the same concentration of NaCl ($p \leq 0.05$).

| **Bacterial Strain** | **---------------------------- NaCl concentration ----------------------------** | | | |
|---|---|---|---|---|
| | 100 g $L^{-1}$ | | 150 g $L^{-1}$ | |
| | **Mean μ** | **Median μ** | **Mean μ** | **Median μ** |
| CaDA4 | 0.1812 | 0.179 abc | 0.124 | 0.126 ab |
| CaDR1 | 0.137 | 0.137 abc | - | - |
| CaDR7 | 0.125 | 0.125 abc | 0.241 | 0.241 a |
| CaPFR3 | 0.159 | 0.166 abc | - | - |
| CaPFR7 | 0.233 | 0.233 a | - | - |
| CaSRM1 | 0.113 | 0.115 abc | 0.102 | 0.095 ab |
| CaSRM12 | 0.074 | 0.071 bc | - | - |
| CaSRM15 | 0.145 | 0.147 abc | - | - |
| CaSRM16 | 0.182 | 0.183 abc | 0.168 | 0.168 a |
| CaSRM18 | 0.147 | 0.147 abc | 0.048 | 0.049 bc |
| CaSRM5 | 0.326 | 0.323 a | - | - |
| CaSRM7 | 0.137 | 0.119 abc | - | - |
| CaSRM8 | 0.165 | 0.165 abc | 0.026 | 0.034 c |
| CaSRM9 | 0.277 | 0.275 ab | 0.100 | 0.102 ab |
| CaSRS1 | 0.080 | 0.080 abc | 0.053 | 0.054 bc |
| CaSRS10 | 0.128 | 0.160 abc | - | - |
| CaSRS4 | 0.179 | 0.175 abc | - | - |
| CaSRS8 | 0.051 | 0.051 c | 0.068 | 0.061 bc |
| CaSS10 | 0.076 | 0.074 bc | 0.016 | 0.016 c |
| CaSS7 | 0.060 | 0.063 c | 0.032 | 0.032 c |

## Sodium perchlorate tolerance

The μ values obtained during the perchlorate tolerance experiment did not meet ANOVA assumptions at concentrations of 10 to 90 g $L^{-1}$ (Tables 4 and 5). As a result, we used the Kruskal-Wallis test and the Dunn test. However, at 110 g $L^{-1}$, we performed ANOVA and the Tukey test (Table 5).

CaSDA4 showed a high μ at NaCl concentrations of 100 and 150 g $L^{-1}$, but could not tolerate more than 30 g $L^{-1}$ $NaClO_4$. This aligns with previous studies that reported a higher tolerance to chloride compared to perchlorate salts (Flores *et al.* 2020; Heinz *et al.* 2019). CaSS10 and CaSRM18 exhibited higher growth rates at 10, 30, 50, and 110 g $L^{-1}$ $NaClO_4$ compared to other strains, but did not perform as well in the NaCl tolerance experiment. This indicates that there is no direct relationship between tolerance to NaCl and $NaClO_4$. Perchlorate is likely hazardous due to its chaotropic and oxidative nature, while NaCl induces osmotic stress (Heinz *et al.* 2021; Saini *et al*. 2023). Therefore, the lack of correlation between the μ values observed in the two experiments may reflect differences in the degree of destabilization caused by these two stressors and in the mechanisms of cell damage caused by each of them.

**Table 4** Specific growth rates (μ) of the bacterial strains in medium containing $NaClO_4$. Strains labeled with different letters indicate significant differences at the same concentration of $NaClO_4$ ($p \leq 0.05$).

| **Bacterial strain** | **---------------------------- $NaClO_4$ concentration ----------------------------** | | | | | |
|---|---|---|---|---|---|---|
| | $10\ g\ L^{-1}$ | | $30\ g\ L^{-1}$ | | $50\ g\ L^{-1}$ | |
| | **Mean μ** | **Median μ** | **Mean μ** | **Median μ** | **Mean μ** | **Median μ** |
| CaDA4 | 0.171 | 0.169 ghi* | 0.147 | 0.162 efg | - | - |
| CaDR1 | 0.156 | 0.167 ghi | 0.171 | 0.169 cdefg | 0.186 | 0.184 bcdefg |
| CaDR7 | 0.095 | 0.099 i | 0.170 | 0.171 cdefg | 0.145 | 0.147 cdefgh |
| CaPFR3 | 0.226 | 0.213 defg | 0.162 | 0.160 defg | 0.117 | 0.112 efgh |
| CaPFR7 | 0.158 | 0.156 ghi | 0.120 | 0.118 g | 0.226 | 0.225 abcde |
| CaSRM1 | 0.389 | 0.377 abcd | 0.534 | 0.546 a | 0.328 | 0.389 abc |
| CaSRM12 | 0.217 | 0.225 fgh | 0.189 | 0.182 cdefg | 0.081 | 0.073 gh |
| CaSRM15 | 0.347 | 0.343 abcdef | 0.178 | 0.174 cdefg | 0.219 | 0.227 abcde |
| CaSRM16 | 0.358 | 0.354 abcdef | 0.231 | 0.224 abc | 0.266 | 0.252 ab |
| CaSRM18 | 0.429 | 0.443 abc | 0.518 | 0.522 a | 0.351 | 0.396 ab |
| CaSRM5 | 0.114 | 0.114 hi | 0.185 | 0.185 bcdef | 0.134 | 0.126 defgh |
| CaSRM7 | 0.251 | 0.262 cdefg | 0.224 | 0.238 abcd | 0.094 | 0.082 fgh |
| CaSRM8 | 0.451 | 0.444 ab | 0.325 | 0.331 ab | 0.259 | 0.251 abc |
| CaSRM9 | 0.237 | 0.229 defg | 0.174 | 0.173 cdefg | 0.144 | 0.138 cdefgh |
| CaSRS1 | 0.212 | 0.213 efgh | 0.213 | 0.220 abcde | 0.042625 | 0.050 h |
| CaSRS10 | 0.374 | 0.371 abcde | 0.263 | 0.276 abc | 0.240825 | 0.213 abcde |
| CaSRS4 | 0.243 | 0.222 efgh | 0.233 | 0.234 abcde | 0.235625 | 0.244 abcd |
| CaSRS8 | 0.260 | 0.254 bcdefg | 0.140 | 0.152 fg | 0.209900 | 0.206 abcdef |
| CaSS10 | 0.560 | 0.537 a | 0.471 | 0.455 a | 0.576775 | 0.574 a |
| CaSS7 | 0.187 | 0.177 ghi | 0.186 | 0.195 bcdefg | 0.042800 | 0.042 h |

**Table 5** Specific growth rates (μ) of the bacterial strains in medium containing 70 110 g $L^{-1}$ $NaClO_4$. Strains labeled with different letters indicate significant differences at the same $NaClO_4$ concentration. For concentrations of 70 and 90 g $L^{-1}$ $NaClO_4$ we performed Kruskal-Wallis and the Dunn statistical test and for 110 g $L^{-1}$ $NaClO_4$ we performed ANOVA and Tukey test ($p \leq 0.05$).

| **Bacterial strain** | **---------------------------- $NaClO_4$ concentration ----------------------------** | | | | | |
|---|---|---|---|---|---|---|
| | 70 g $L^{-1}$ | | 90 g $L^{-1}$ | | 110 g $L^{-1}$ | |
| | **Mean μ** | **Median μ** | **Mean μ** | **Median μ** | **Mean μ** | **Median μ** |
| CaDR1 | 0.545 | 0.536 a* | 0.122 | 0.107 efgh | - | - |
| CaDR7 | 0.240 | 0.230 cdef | 0.161 | 0.160 bcd | - | - |
| CaPFR3 | 0.152 | 0.150 fghi | 0.131 | 0.125 defgh | 0.152 ab | 0.162 |
| CaPFR7 | 0.313 | 0.320 abc | 0.303 | 0.286 ab | - | - |
| CaSRM1 | 0.077 | 0.082 ghi | - | - | - | - |
| CaSRM12 | 0.287 | 0.291 bcde | 0.227 | 0.230 abcd | 0.105 b | 0.124 |
| CaSRM15 | 0.317 | 0.306 abcd | 0.203 | 0.206 abcde | 0.259 a | 0.245 |
| CaSRM16 | 0.381 | 0.366 ab | 0.245 | 0.248 abc | 0.140 ab | 0.147 |
| CaSRM18 | 0.175 | 0.175 defg | 0.190 | 0.175 bcd | 0.176 ab | 0.223 |
| CaSRM5 | 0.164 | 0.155 efgh | 0.290 | 0.29150 a | 0.104 b | 0.096 |
| CaSRM7 | 0.138 | 0.137 fghi | 0.058 | 0.04885 gh | 0.151 ab | 0.131 |
| CaSRM8 | 0.155 | 0.155 fghi | 0.183 | 0.18350 bc | 0.152 ab | 0.154 |
| CaSRM9 | 0.356 | 0.316 abc | 0.272 | 0.27350 ab | 0.094 b | 0.086 |
| CaSRS1 | 0.034 | 0.033 hi | 0.023 | 0.02255 h | 0.055 b | 0.042 |
| CaSRS10 | 0.298 | 0.288 bcd | 0.193 | 0.18595 bc | 0.095 b | 0.097 |
| CaSRS4 | 0.379 | 0.380 ab | 0.237 | 0.23720 abc | 0.138 ab | 0.127 |
| CaSRS8 | 0.096 | 0.101 ghi | 0.039 | 0.03830 h | 0.066 b | 0.067 |
| CaSS10 | 0.289 | 0.282 bcde | 0.222 | 0.22065 abcd | - | - |
| CaSS7 | 0.027 | 0.028 i | 0.082 | 0.08610 fgh | 0.137 ab | 0.132 |

## Perchlorate reduction

During anaerobic growth on $NaClO_4$ as electron acceptor, the bacterial strains CaSRS1, CaSRS8, CaSRM5, CaSRM7, CaSRM12, and CaSRM16 formed cell aggregates that could not be disrupted even with intense vortexing (Fig. 3). Microscopic aggregates were also observed for the other strains (Fig. 4). This agrees with previous studies that reported aggregate formation as a response to perchlorate toxicity (Heinz et al., 2019). With the exceptions of CaSS7 and CaSRM7, all microorganisms produced detectable biomass (Fig 5A). Among these, CaDR1 exhibited the highest biomass, and CaDR1 and CaDR7 demonstrated the highest luciferase luminescence in the ATP assay. This assay indicated that all strains showed luciferase luminescence, demonstrating their viability in a medium where perchlorate served as the sole electron acceptor (Fig 5B). The ability to not only tolerate perchlorate but also utilize it for respiration is particularly significant in astrobiology, especially in the context of an anoxic Mars with brines rich in perchlorate salts. These organisms could serve as models for studying Mars's habitability and raise concerns about interplanetary contamination. Their capacity to withstand and respire perchlorate makes them potential contaminants of Mars. Furthermore, *Bacillus* species can form endospores, enhancing their resilience to conditions found on Mars and in spacecraft (Mettler et al., 2023).

**Fig. 3** Cell aggregates formed by CaSRS1, CaSRS8, CaSRM5, CaSRM7, CaSRM9, CaSRM12, and CaSRM16 during anaerobic growth using perchlorate as sole electrons acceptor.

**Fig. 4** Microscopic visualization of the bacterial strains after 14 days in anaerobic medium with sodium perchlorate as sole electrons acceptor. CaDR1, CaDR7, CaSDA4, CaPFR3, CaPFR7, CaSS7, CaSS10, CaSRS1, CaSRS4, CaSRS8, CaSRS10, CaSRM1, CaSRM5, CaSRM7, CaSRM8, CaSRM9, CaSRM12, CaSRM15, and CaSRM18 (A, B, C, D, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, and T respectively).

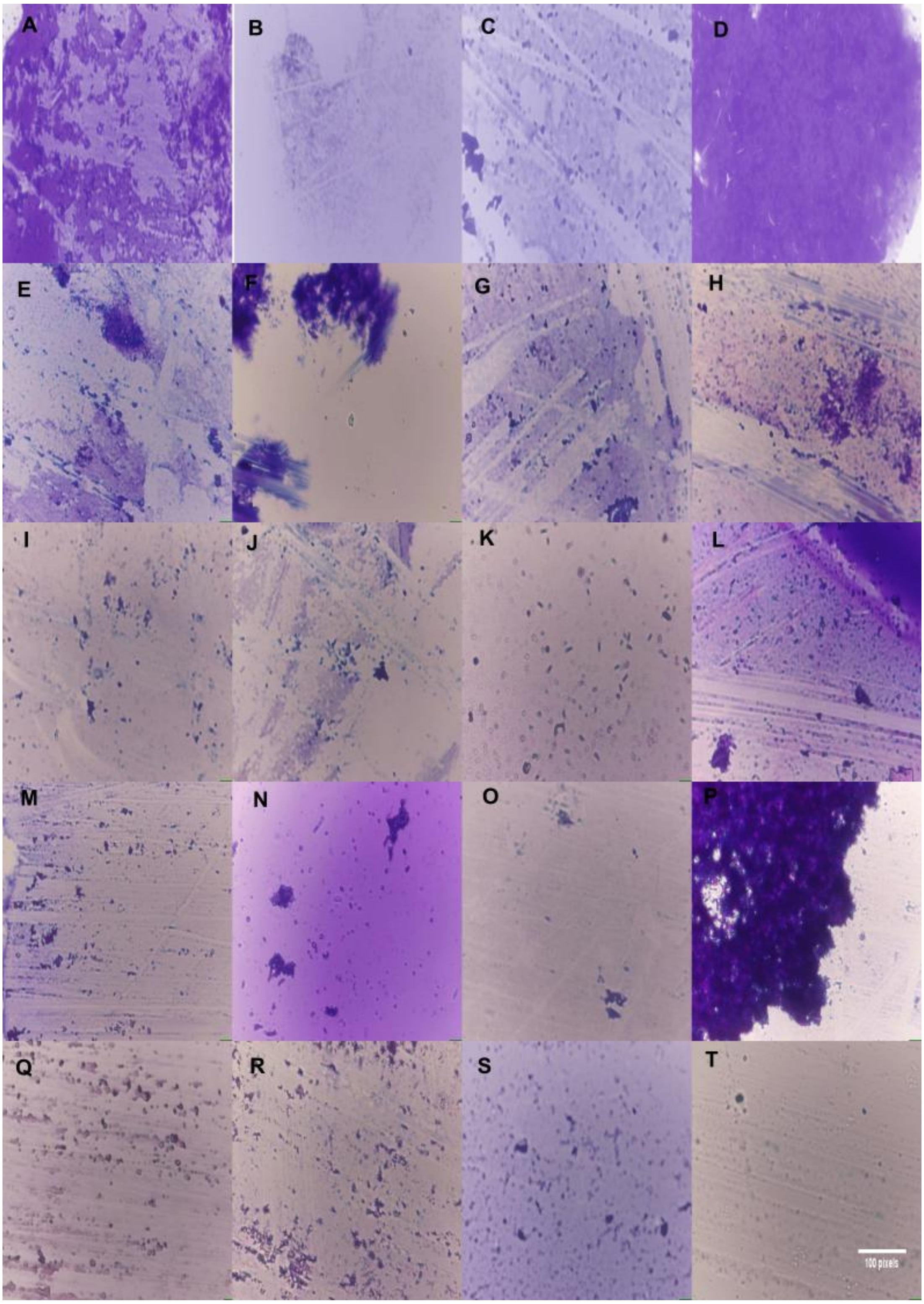
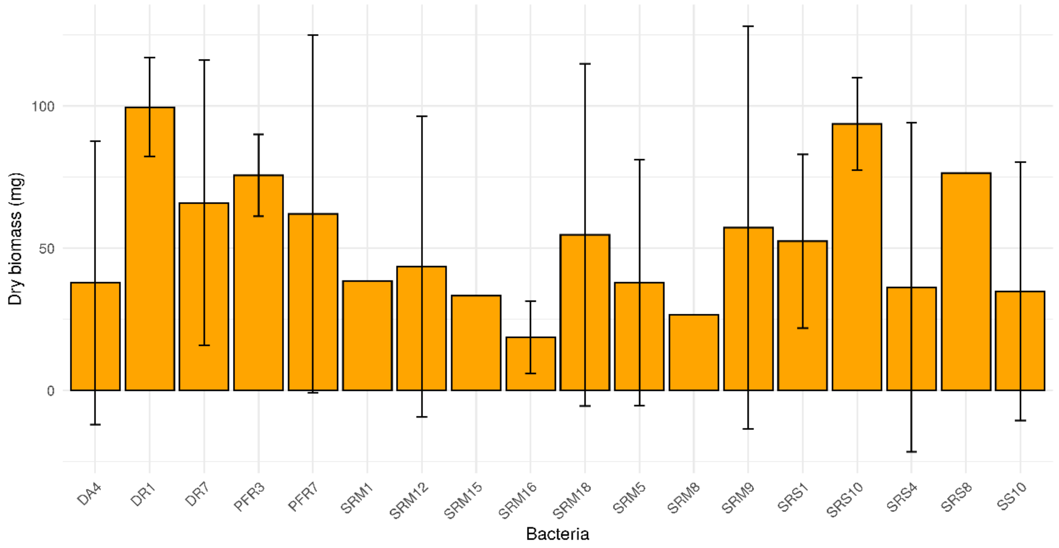
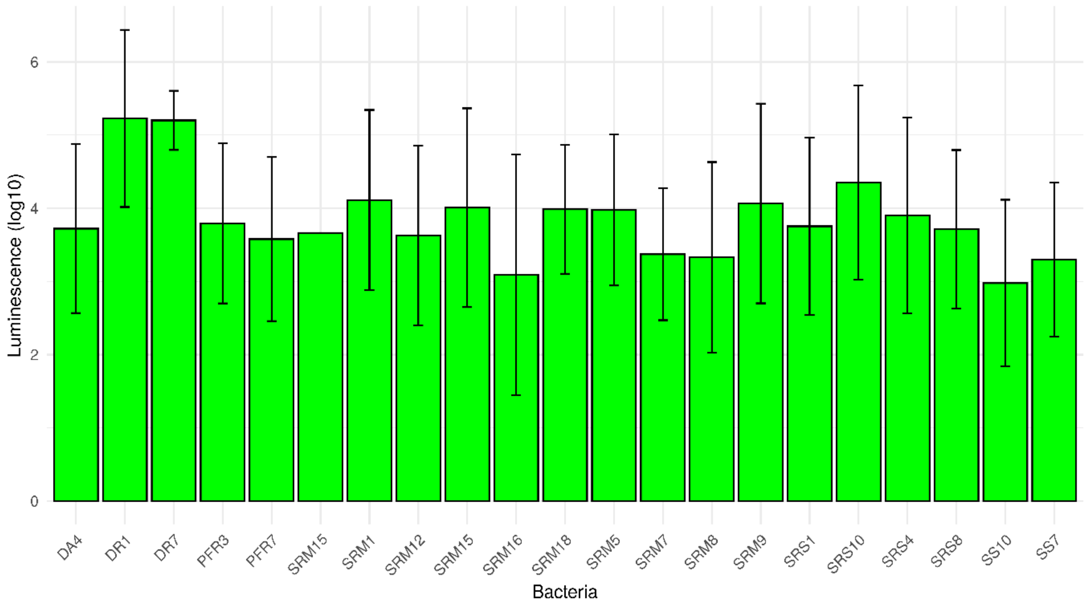


**Fig. 5** Growth and ATP-derived luminescence of the bacterial strains in medium with perchlorate as sole electrons acceptor. A: dry biomass of the strains in 15 mL of medium after 14 days of incubition; B: ATP assay using luciferin/luciferase probe.

A- Dry biomass of the strains in 15 mL of medium after 14 days of incubation



B- ATP assay using luciferin/luciferase probe



### Biosurfactant production and growth on hydrocarbon

In the drop collapse test, CaPFR3, CaPFR7, CaSRM5, CaSRM8, and CaSRM18 showed positive results and were further tested for reduction of surface tension in TSB medium (Table 10). The surface tension of the growth medium was 72. All the above isolates could reduce the surface tension of the medium. The lowest surface tension was obtained in the medium inoculated with CaSRM18 (27.716 +/- 0.018). This biosurfactant producing bacterium was the only able to growth in MSM medium containing hydrocarbon as the sole carbon source (see below). Other bacteria capable of growing using hydrocarbon as the sole carbon source (CaSDR1, CaSDR7, CaSRS10, and CaSRM15) did not produce biosurfactant in the tested conditions. Halotolerant *Bacillus* are known for producing biosurfactants. For example, a marine *Bacillus licheniformis* could reduce surface tension of the growth medium by 31.4% and degrade engine oil (Nayak et al. 2020). Accordingly, *Bacillus halotolerans*, isolated from metal-contaminated soil, produces biosurfactants with potential antioxidant, antimicrobial, and anticancer properties (Etemadzadeh *et al*. 2024).

Biosurfactants have various applications beyond the oil industry, including use in detergents, food, cosmetics, pharmaceuticals, agriculture, and nanotechnology (Sarubbo et al. 2022). Therefore, even strains that cannot use hexadecane or other hydrocarbons as the sole carbon source can be a source of biosurfactants for a variety of applications.

**Table 6** Biosurfactant Production by the halotolerant bacterial strains. Surface tension was measured only for bacteria that showed positive results in the drop collapse test. Additionally, growth in MSM medium containing hexadecane as the sole carbon source was assessed.

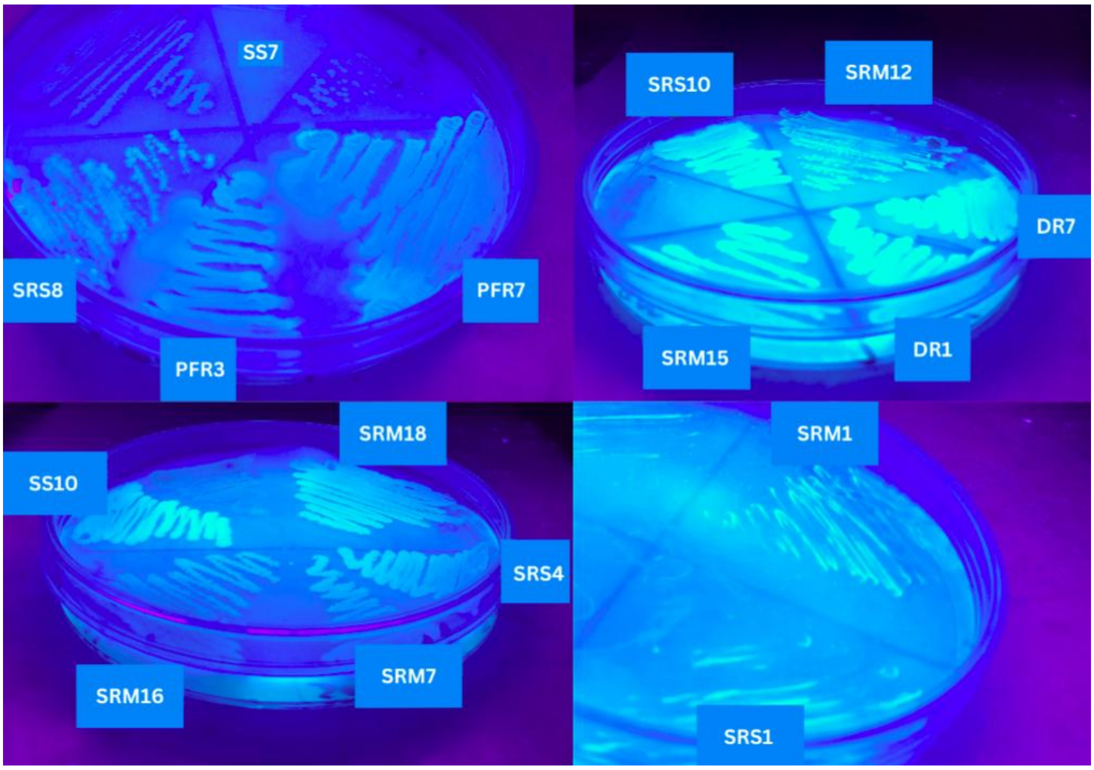
*NE = Not Evaluated

| Strain | Drop collapse | Surface tension | Growth on hydrocarbon |
|---|---|---|---|
| CaSDR1 | - | NE | + |
| CaSDR7 | - | NE | + |
| CaSDA4 | - | NE | - |
| CaPFR3 | + | 29.187 +/- 0.025 | - |
| CaPFR7 | + | 30.350 +/- 0.023 | - |
| CaSRS1 | - | NE | - |
| CaSRS4 | - | NE | - |
| CaSRS8 | - | NE | - |
| CaSRS10 | - | NE | + |
| CaSS7 | - | NE | - |
| CaSS10 | - | NE | - |
| CaSRM1 | - | NE | - |
| CaSRM5 | + | 29.511 +/- 0.029 | - |
| CaSRM7 | - | NE | - |
| CaSRM8 | + | 45.910 +/- 0.029 | - |
| CaSRM9 | - | NE | - |
| CaSRM12 | - | NE | - |
| CaSRM15 | - | NE | + |
| CaSRM16 | - | NE | - |
| CaSRM18 | + | 27.716 +/- 0.018 | + |

## Synthesis of exopolysaccharides

Sixteen among the 20 bacterial strains showed positive results for exopolysaccharide synthesis (Figure 6). The production of exopolysaccharide (EPS) aligns with the observations of aggregate formation during perchlorate reduction experiments, as these polymers play a crucial role in cellular aggregation and adherence. Halophilic and halotolerant bacteria synthesize exopolysaccharides (EPS) as a survival mechanism when exposed to harsh conditions. These polymers can exhibit biosurfactant properties and, therefore, contribute to bioremediation (Ibrahim et al., 2020). In addition to bioremediation, EPS also finds applications in the food, pharmaceutical, and agricultural industries. Moreover, EPS is utilized in waste treatment due to its metal chelation, flocculation, and colloid formation properties. Research has shown that EPS produced by *Bacillus* spp. isolated from mangrove ecosystems demonstrated antimicrobial, cytotoxic, antioxidant, and anti-inflammatory activities, highlighting the importance of studying EPS-producing strains from these and other underexplored ecosystems (Díaz-Cornejo et al., 2023). Additionally, exopolysaccharides produced by this genus were capable of inhibiting biofilm formation by *Staphylococcus aureus*, a pathogen associated with bovine mastitis in veterinary medicine (Sabino *et al.* 2023).

**Fig. 6** Exopolysaccharide synthesis by the halotolerant bacterial strains. Fluorescence emission upon exposure to UV-C light indicates positive result.



## Enzymatic production

All the bacterial isolates produced at least one of the three enzymes evaluated (Table 11, S1, S2, and S3). Lipase and protease were produced more frequently (16 and 14 out of 20 bacterial isolates, respectively). In contrast, only 6 isolates produced amylase. Notably, CaSRM5 and CaSRM16 produced all three enzymes, while five isolates produced just one enzyme: CaSDA4 and CaSRS8 produced proteases; CaSRM8 produced amylases; and CaSRM18, CaSS7, and CaSS10 produced lipases.

The production of hydrolases by salt-tolerant bacteria was reported by Drissi Kaitouni et al. (2020), who isolated bacteria from a salt mine and two salt marshes. From 227 bacteria collected, they found that 189 synthesized hydrolases, including amylases and proteases. Additionally, Fatholahpoor et al. (2020) isolated salt-tolerant bacteria from the Eshtehard Desert in Iran, and reported that out of 32 strains, 18 exhibited lipase activity, 12 showed protease activity, and 11 had amylase activity. These enzymes, aside from their role in wastewater treatment, have applications in various industries, such as food, pharmaceuticals, and detergents, among others (Mesbah 2022; Mokasheet al. 2018; Qiu et al. 2021).

**Table 7** Production of amylases, lipases and proteases by the strains.

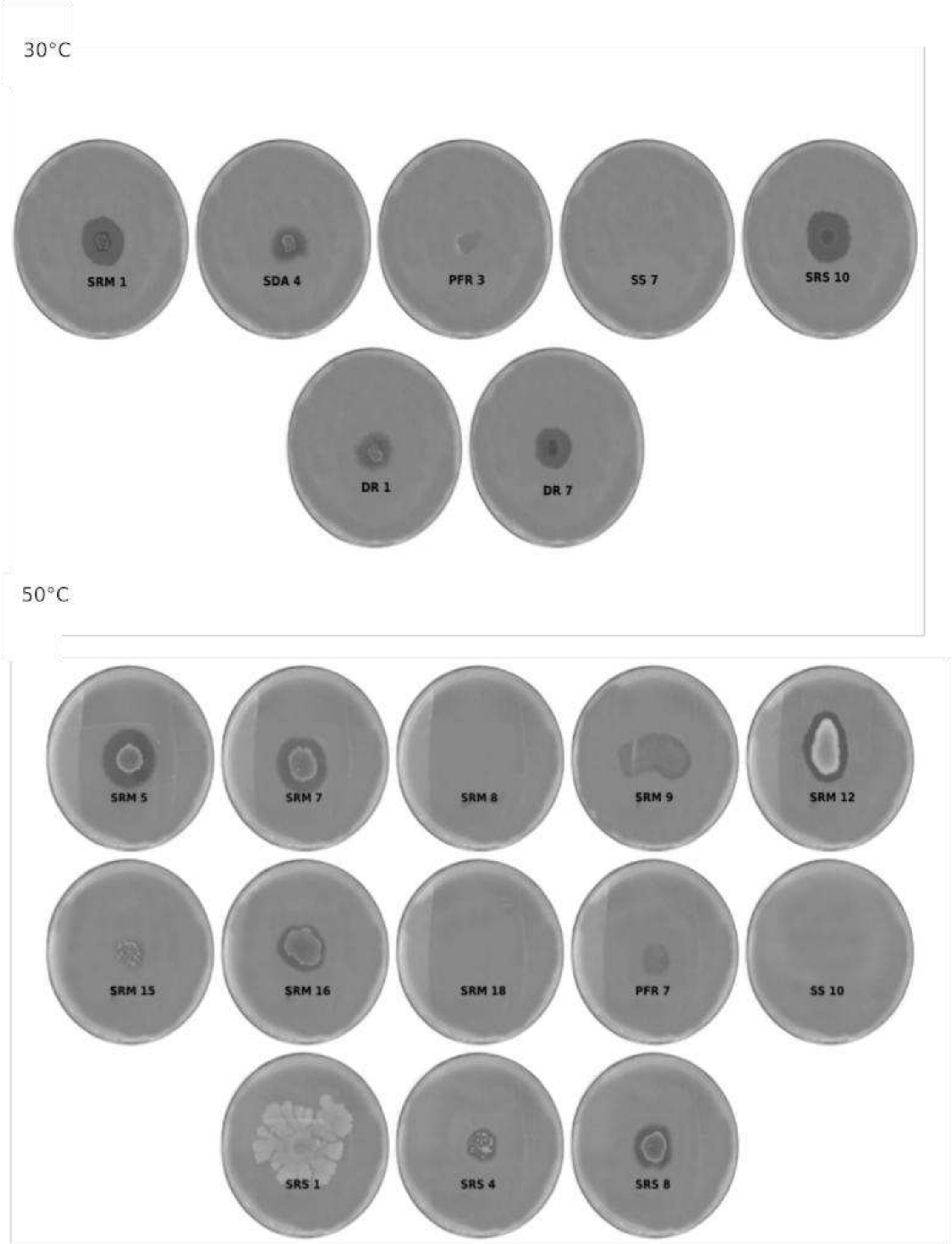
| Strain | amylases | lipases | proteases |
|---|---|---|---|
| CaDR1 | - | + | + |
| CaDR7 | - | + | + |
| CaSDA4 | - | - | + |
| CaPFR3 | + | + | - |
| CaPFR7 | - | + | + |
| CaSRM1 | + | - | + |
| CaSRM5 | + | + | + |
| CaSRM7 | - | + | + |
| CaSRM8 | + | - | - |
| CaSRM9 | - | + | + |
| CaSRM12 | - | + | + |
| CaSRM15 | - | + | + |
| CaSRM16 | + | + | + |
| CaSRM18 | - | + | - |
| CaSRS1 | + | + | - |
| CaSRS4 | - | + | + |
| CaSRS8 | - | - | + |
| CaSRS10 | - | + | + |
| CaSS7 | - | + | - |
| CaSS10 | - | + | - |

## CONCLUSION

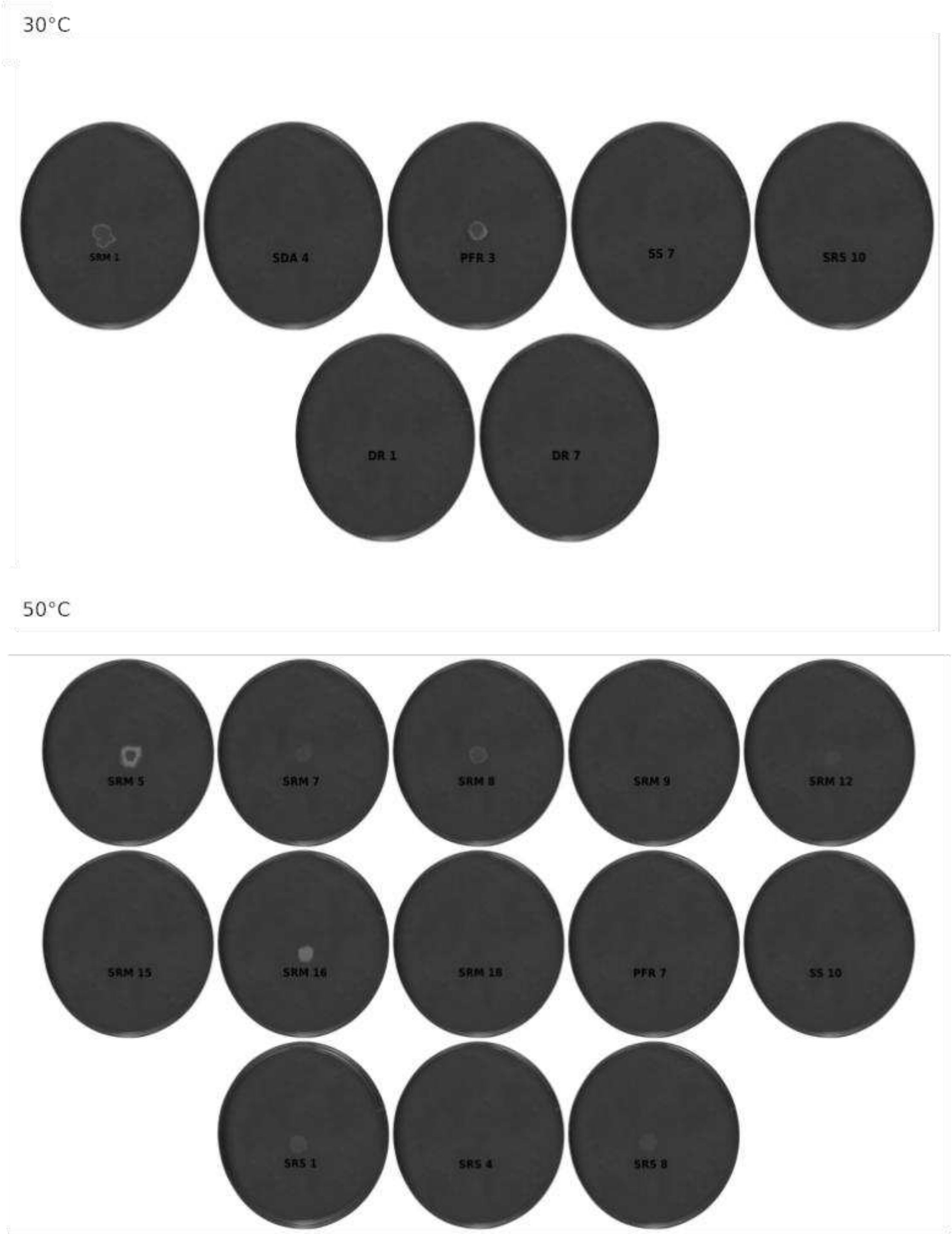
Brazil possesses significant potential for a vast microbial biodiversity with various applications. Our study focused on the biotechnological and astrobiological applications of halotolerant bacteria isolated from different habitats in a microbiologically underexplored region (Camocim, CE). These microorganisms can tolerate high concentrations of NaCl and perchlorate, a toxic salt commonly found on Mars. Moreover, they can utilize sodium perchlorate as a sole electron acceptor in anaerobic conditions, making them excellent astrobiological models and candidates for survival experiments under simulated anoxic Martian environments. In terms of biotechnological applications, five of the 20 microorganisms produced biosurfactants, while 16 produced exopolysaccharides, both of which have numerous industrial and biotechnological applications. Additionally, these microorganisms produce the enzymes lipases, proteases, and amylases, which are applicable in various industrial sectors.

This study highlights the importance of improving prospection efforts to unravel the microbial diversity, particularly of extremophiles, and lays the groundwork for future research. Upcoming studies should focus on using omics technologies to better understand the mechanisms behind perchlorate tolerance and reduction by these organisms, as well as assess their specific industrial applications.

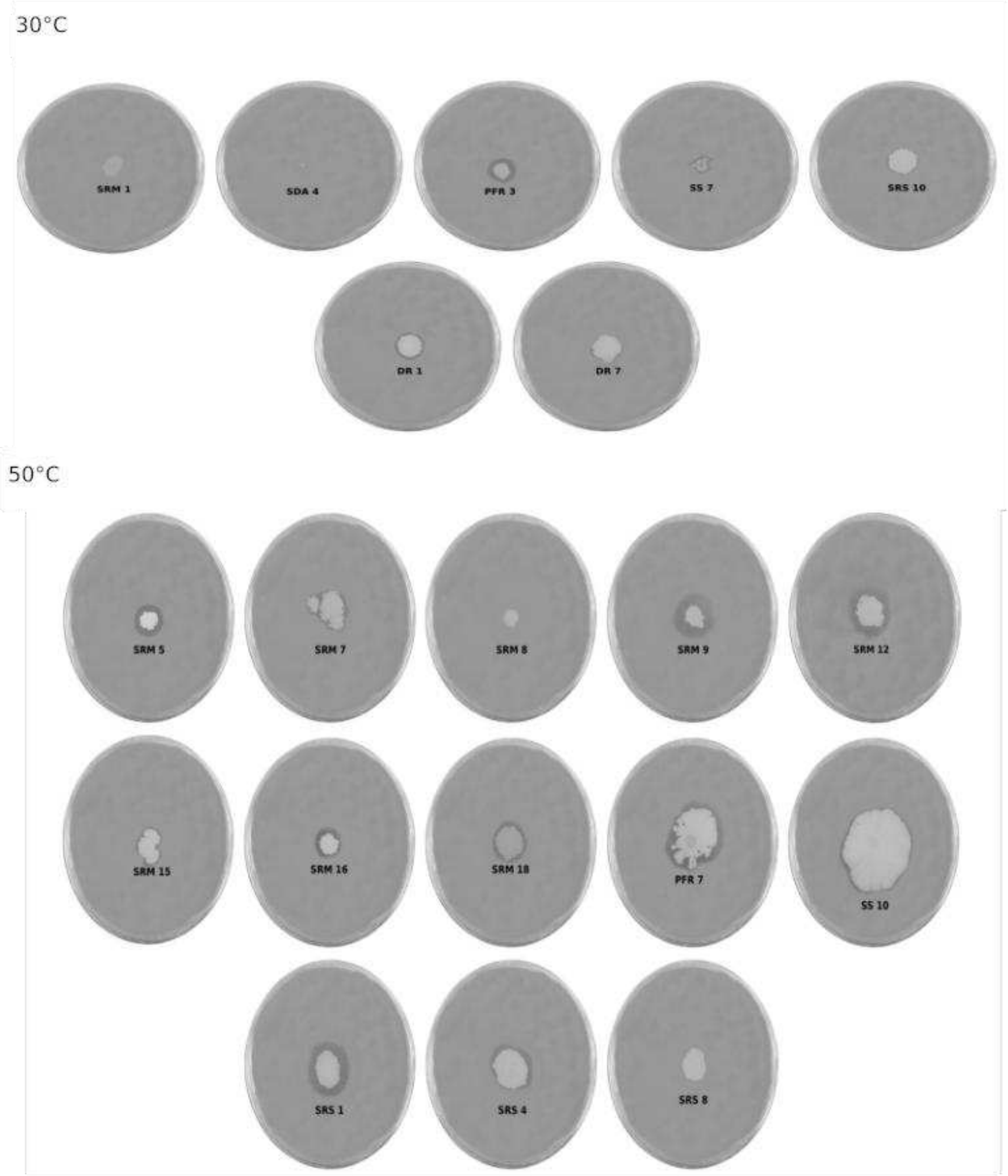
**S1** Protease production by the strains. Halos indicate positive results.



**S2** Amylase production by the strains. Halos indicate positive results.



**S3** Lipase production by the strains. Halos indicate positive results.



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# CHAPTER III: DELVING INTO ADAPTIVE MECHANISMS OF HALOTOLERANT BACTERIA THROUGH ADAPTIVE LABORATORY EVOLUTION

## INTRODUCTION

Salt-tolerant bacteria possess a range of adaptive mechanisms that enable them to thrive in saline environments. The most reported mechanisms are "salt-in" and "salt-out." The salt-in mechanism equilibrates the ionic concentration within the cell, preventing damage from excessive sodium ($Na^+$) levels. In contrast, the salt-out mechanism helps prevent water loss due to osmosis by accumulating compatible solutes inside the cell (Mukhtar *et al.* 2020). Understanding the adaptive mechanisms of halotolerant bacteria is crucial for future research, as these microorganisms can be utilized in activities such as bioremediation of saline environments and the treatment of saline wastewater (Pourfadakari *et al.* 2021; Tan *et al.* 2019). Additionally, this knowledge can provide insights into how organisms may adapt to saline extraterrestrial environments, such as those found on the icy moons Europa and Enceladus, as well as on past and present Mars (Schultz *et al.* 2023).

Adaptive laboratory evolution (ALE) is a method used to study adaptative mechanisms in light of evolution under specific conditions, such as high salinity, low or high temperature, etc. By cultivating microorganisms in controlled environments over a defined period - ranging from weeks to months or even years - we can select for a specific phenotype of interest. Furthermore, next-generation DNA sequencing (NGS) and bioinformatics analysis enable us to identify the genetic changes responsible for the new phenotype (Dragosits and Mattanovich 2013). As a result, we can uncover the mutations necessary to achieve a particular phenotype. These mutations may include single nucleotide polymorphisms (SNPs), deletions (DELs), mobile elements (MOBs), or insertions (INS) (Phaneuf *et al.* 2019).

These genetic modifications can lead to changes in physiological responses to environmental stress, with one common response being the alteration of the fatty acid composition (Wang *et al.* 2020). For example, *Rhodococcus erythropolis* increases the percentage of polyunsaturated fatty acids, particularly eicosapentaenoic acid (C20:5ω3), arachidonic acid (C20:4ω6), and docosapentaenoic acid (C22:5ω3), when exposed to saline stress. These changes reduce the negative charge in ion channels, helping to repel $Na^+$ and alleviate osmotic pressure (De Carvalho *et al.* 2014). Conversely, *Halomonas alkaliphila* increases the percentage of saturated fatty acids in response to salt stress, which enhances membrane rigidity and resistance to osmotic pressure (El Halmouch, 2019). While these modifications have been observed, the impact of adaptive laboratory evolution on these physiological and compositional responses remains uncertain.

Some studies have explored how adaptive laboratory evolution can enhance industrially relevant phenotypes under saline stress. For instance, *Bacillus siamensis* 072 demonstrated improved macrolactin production after undergoing genetic adaptation to saline conditions. This improvement is likely due to a mutation in the *hisD* gene, which encodes histidine dehydrogenase (Gan *et al*. 2022). Additionally, lactic acid bacteria showed increased lactic acid production when grown in hypersaline seaweed hydrolysate. This latest study also examined the mutations that contributed to the salt tolerance phenotype, identifying alterations in genes associated with cellular ion balance, cell membrane composition, and proteins that function as regulators (Papadopoulou *et al*. 2023).

Halophiles and halotolerants are capable of surviving in high concentrations of other salts, not just sodium chloride (NaCl). These organisms can endure different concentrations of perchlorate salts, despite the high toxicity of this compound. Interestingly, halophiles and halotolerants not only survive in the presence of perchlorate but can also utilize this salt as electron acceptor in anoxic conditions (Heinz *et al.* 2019; Oren *et al*. 2014). This is particularly noteworthy, given the current lack of oxygen on Mars and the presence of perchlorate on its surface. However, there is limited knowledge regarding studies that have employed Adaptive Laboratory Evolution to induce mutations that enhance growth in perchlorate-rich environments.

Although the mechanisms of salt tolerance are known, it is not of our knowledge studies that have explored the Adaptive Laboratory Evolution (ALE) approach to investigate its effects on perchlorate tolerance and fatty acid profile in prokaryotes. Therefore, this study aims to use ALE to explore how random mutations influence sodium perchlorate tolerance and the physiological stress response, with particular emphasis on changes in the cell's fatty acid profile.

## MATERIALS AND METHODS

### Selection of the bacterial strains

The bacterial strains *Bacillus glycinefermentans* S1C5 and *Bacillus sp.* CaSRM5 are part of the collection culture of Laboratório de Biotecnologia e Biodiversidade para o Meio Ambiente, Universidade Federal de Viçosa, Viçosa, Brazil. S1C5 was isolated from Trindade island, and CaSRM5 was isolated from the Mangrove of Camocim, Ceará State, both underexplored sites for microbial diversity from Brazil. They were selected based on previous studies on NaCl tolerance. S1C5 was chosen due to the results of a previous adaptation experiment, during which it presented the best results compared to other strains (unpublished data). CaSRM5 was selected based on its high specific growth rate at 100 g $L^{-1}$ NaCl.

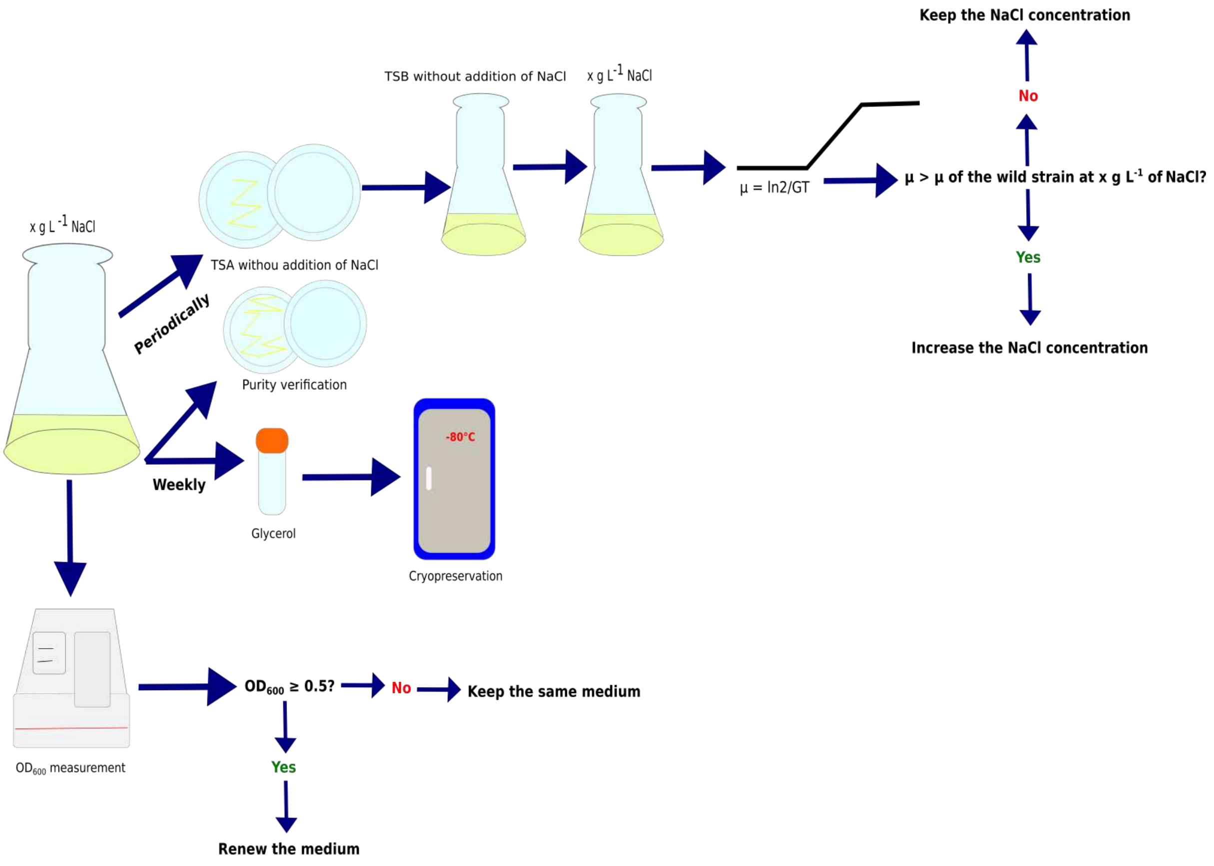
### Adaptive laboratory evolution

Microorganisms were grown in 25 mL of TSB in 125 mL Erlenmeyer flasks at 30 °C and 200 rpm, with salt concentrations increasing from 100 to 200 g $L^{-1}$ (100, 120, 150, 180, and 200 g $L^{-1}$) over the time required for the laboratory adaptive evolution experiment. Growth was monitored every 12 hours by optical density at 600 nm ($OD_{600}$). The culture medium was renewed when the $OD_{600}$ reached a minimum of 0.5, transferring 1% of the flask volume to fresh medium. The NaCl concentration was increased when the evolved strain exhibited a higher specific growth rate (µ) than the parental strain at the actual salt concentration. The specific growth rate (µ) was determined based on the growth curves, as described in the Growth Kinetics section. Every week, aliquots from each culture were collected for cryopreservation in 25% glycerol at -80°C. Additionally, to verify culture purity, samples were plated on TSA weekly (Fig. 1). The experiment was conducted with three Erlenmeyer flasks (each one as an evolutionary lineage) per parental strain and lasted 250 days.

### Growth Kinetic

Growth curves were obtained at the beginning of the adaptive evolution experiments (at 0 and 100 g $L^{-1}$ NaCl), periodically throughout the experiment to compare the growth of evolved and non-evolved strains, and again after 250 days to compare the wild-type and the final evolved strains. The data were used to calculate the generation time () and the specific growth rate (µ) of the bacterial strains. Additionally, the evolved strains were assessed for their ability to grow in Tryptic Soy Broth (TSB) without additional NaCl (TSB and TSA contains 5 g $L^{-1}$ NaCl). For the growth curve experiments, cultures were streaked onto TSA. The inoculum was prepared transferring one isolated colony to 25 mL of TSB. These cultures were incubated at 30°C with shaking at 200 rpm for 24 hours. An aliquot was then transferred in triplicate to TSB medium with the corresponding NaCl concentrations in order to achieve an initial optical density of 0.2. The cultures were maintained at 30°C and 200 rpm, with periodic measurements taken to monitor bacterial growth (Fig. 1). The generation time was calculated based on the specific growth rate using the formula $GT = \ln 2/\mu$. To compare the generation time of wild and evolved strains at the maximum NaCl concentration tolerated by the wild strain, we conducted, after 250 days of adaptive evolution, an Analysis of Variance (ANOVA), followed by Tukey's post-hoc test for data that met ANOVA assumptions. For concentrations that did not meet these assumptions, we used the Kruskal-Wallis test, followed by Dunn's post-hoc test. Statistical analyses and graphical representations were performed using R software. The periodicity of the growth curves was determined by the stabilization of the $OD_{600}$ in the tested NaCl concentration.

**Fig.1** Adaptive laboratory evolution experimental design. Salt concentrations increasing from 100 to 200 g $L^{-1}$ (100, 120, 150, 180, and 200 g $L^{-1}$)



### Effects of ALE on perchlorate tolerance

The experiments were conducted using a 96-well microplate to evaluate the growth of both parental and evolved isolates, the latter having been previously subjected to adaptive laboratory evolution (ALE) with progressively increasing NaCl concentrations. Microorganisms were cultured at 30°C in Tryptic Soy Broth (TSB) supplemented with sodium perchlorate ($NaClO_4$) at fixed concentrations of 50, 70, and 90 g $L^{-1}$. The growth performance of the parental and evolved strains was directly compared under these varying perchlorate concentrations. The data were recorded using Gen5 software. To assess perchlorate tolerance, we calculated the specific growth rate ($\mu$) of the bacterial strains. Comparisons of specific growth rates among strains were performed using ANOVA (Analysis of Variance) followed by Tukey's post-hoc test when ANOVA assumptions were met. For conditions that did not meet these assumptions, the Kruskal-Wallis test was applied, followed by Dunn's post-hoc test. Statistical analyses and graphical representations were generated using R software.

### Effects of ALE on fatty acids profile

Evolved and parental strains were cultivated on TSA, both without and with 100 g $L^{-1}$ additional NaCl, to evaluate eventual changes in fatty acids profile following ALE under normal and saline conditions. The analysis was performed using the fatty acid methyl ester (FAME) analysis protocol recommended by MIDI Inc., Newark, Delaware. Subsequently, the extracted FAME were analyzed using MIDI Sherlock® software in an Agilent 7890A gas chromatograph, using the RTSB method and library.

## RESULTS AND DISCUSSION

### Kinetic evaluations

For the wild strain S1C5(W), the maximum NaCl concentration tolerated was 150 g $L^{-1}$ (Table 1), with a lag phase of 43 hours and a specific growth rate ($\mu$) of 0.158 $h^{-1}$. This value was statistically equal to that of S1C5(1) and S1C5(3) but lower than that of S1C5(2) at the same NaCl concentration (Fig. 2). The evolved strains S1C5(1) and S1C5(3) tolerated up to 180 g $L^{-1}$, with specific growth rates of 0.117 $h^{-1}$ and 0.103 $h^{-1}$, respectively, and a lag phase of 24 hours. In contrast, strain S1C5(2) was able to grow in the medium with the highest NaCl concentration tested (200 g $L^{-1}$), with a shorter lag phase of only 8 hours and a specific growth rate of 0.139 $h^{-1}$.

The wild strain SRM5 tolerated up to 120 g $L^{-1}$ with a specific growth rate of 0.189 $h^{-1}$. The evolved strains SRM5(2) and SRM5(3) withstood up to 150 g $L^{-1}$, with specific growth rates of 0.129 $h^{-1}$ and 0.092 $h^{-1}$, respectively, and a lag phase of 57 hours. Strain SRM5(1) withstood up to 120 g $L^{-1}$, similar to the wild strain, but displayed a higher specific growth rate of 0.223 $h^{-1}$ and a longer lag phase (14 x 20 hours for SRM5(W) and SRM5(1), respectively). The $\mu$ of SRM5(1) was statistically equal to that of the parental strain SRM5(W), while that of SRM5(2) and SRM5(3) was significantly higher at 120 g $L^{-1}$ NaCl (Fig. 2).

Gan *et al*. (2022) also observed the improvement in salt tolerance in a study after conducting adaptive laboratory evolution (ALE) experiments with *Bacillus siamensis* 072. The evolved strain demonstrated a 32.03% increase in tolerance to NaCl compared to the wild strain at 60 g $L^{-1}$ NaCl. The experiment stopped at 90 g $L^{-1}$ NaCl, where the evolved strain exhibited minimal cell growth. Another study found that the lactic acid bacteria *Lactobacillus plantarum* and *Enterococcus faecium* also enhanced their NaCl tolerance through ALE experiments. The tolerance of *L. plantarum* improved from 55 g $L^{-1}$ to 71 g $L^{-1}$, while *E. faecium* increased from 40.6 g $L^{-1}$ to 71 g $L^{-1}$ (Papadopoulou *et al*. 2023).

In TSB without any added NaCl, both wild strains, S1C5(W) and SRM5(W), showed a higher specific growth rate compared to the evolved strains (Table 2). We hypothesize that the ALE process resulted in a greater dependence on NaCl in the evolved strains. This finding contrasts with results reported by Dhar *et al*. (2011), where ALE experiments with *Saccharomyces cerevisiae* indicated that the evolved strains exhibited an increased growth rate in media without added NaCl.

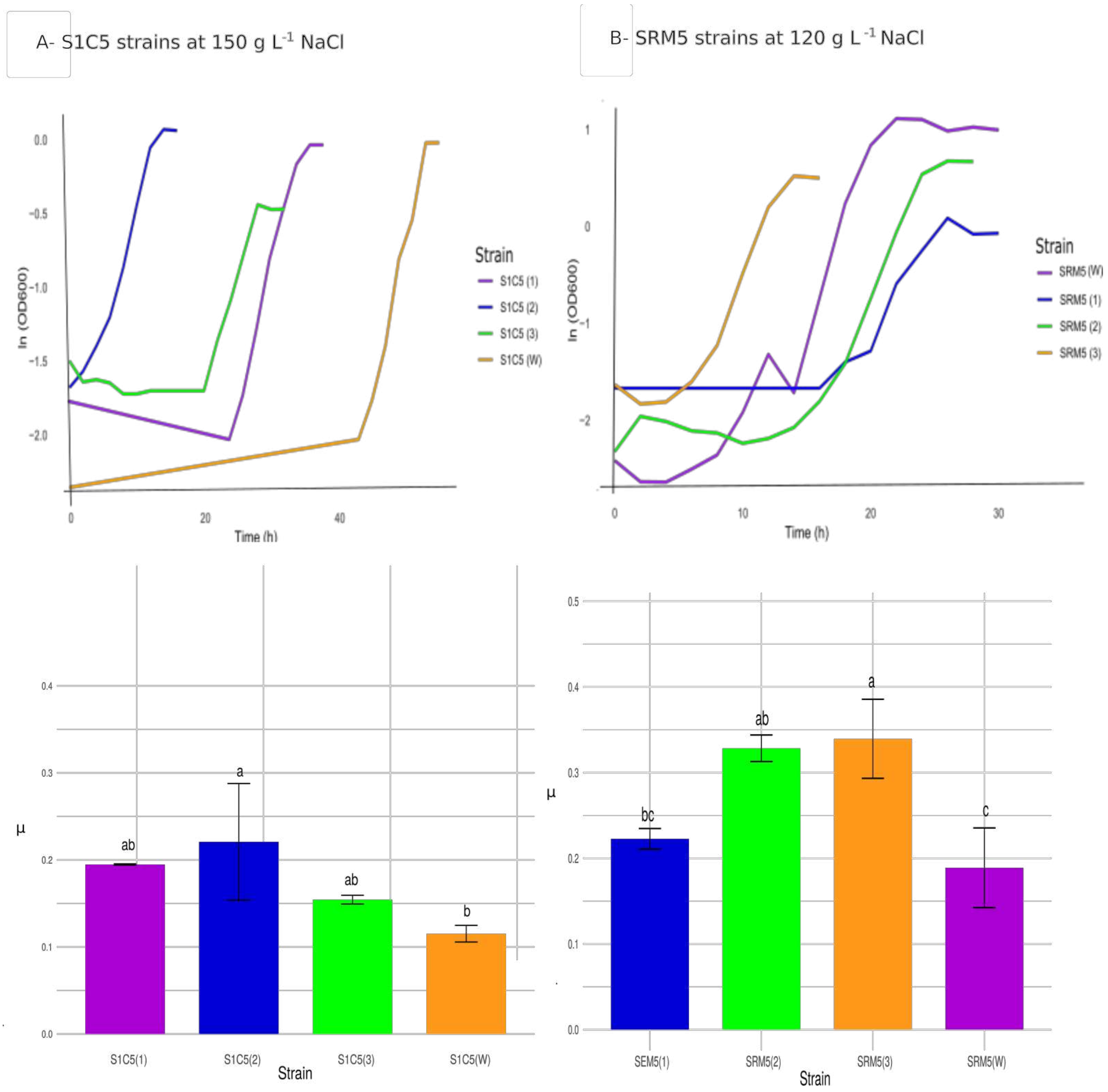
**Table 1** Maximum NaCl concentrations supported by wild-type and evolved strains, and their respective growth kinetic parameters in TSB contained different NaCl concentrations.

| **Strain** | **Maximum NaCl concentration withstood** | **Lag phase duration in the maximum concentration withstood** | **Average μ in the maximum concentration withstood** |
|---|---|---|---|
| S1C5 (W) | 150 g $L^{-1}$ | ~43 hours | 0.15785 $h^{-1}$ |
| S1C5 (1) | 180 g $L^{-1}$ | ~24 hours | 0.117 $h^{-1}$ |
| S1C5 (2) | 200 g $L^{-1}$ | ~8 hours | 0.1387 $h^{-1}$ |
| S1C5 (3) | 180 g $L^{-1}$ | ~24 hours | 0.103 $h\text{-}^{1}$ |
| SRM5(W) | 120 g $L^{-1}$ | ~14 hours | 0.1894 $h^{-1}$ |
| SRM5(1) | 120 g $L^{-1}$ | ~20 hours | 0.2227 $h^{-1}$ |
| SRM5(2) | 150 g $L^{-1}$ | ~57 hours | 0.1294 $h^{-1}$ |
| SRM5(3) | 150 g $L^{-1}$ | ~57 hours | 0.09175 $h^{-1}$ |

**Table 2** Specific growth rate of the wild and evolved strains in TSB without the addition of NaCl.

| **Strain** | **Average μ in 0 g $L^{-1}$ NaCl** |
|---|---|
| S1C5 (W) | 0.779 $h^{-1}$ |
| S1C5 (1) | 0.339 $h^{-1}$ |
| S1C5 (2) | 0.380 $h^{-1}$ |
| S1C5 (3) | 0.314 $h^{-1}$ |
| SRM5(W) | 0.582 $h^{-1}$ |
| SRM5(1) | 0.309 $h^{-1}$ |
| SRM5(2) | 0.308 $h^{-1}$ |
| SRM5(3) | 0.291 $h^{-1}$ |

**Fig. 2** Growth curves and specific growth rate (A and B) of the wild and evolved strains in the maximum NaCl concentration withstood by the wild strain. Strains labeled with different letters indicate significant differences ($p \leq 0.05$).



## Effects of ALE on perchlorate tolerance

At a sodium perchlorate concentration of 50 g $L^{-1}$, the highest maximum $OD_{600}$ was reached by strains S1C5(2) and SRM5(W) (~ 0.9) (Figure 3A). The wild strains' specific growth rate (μ) at this concentration was statistically equal to that of the evolved strains (Figure 4A). At 70 g $L^{-1}$ $NaClO_4$, the differences in the maximum $OD_{600}$ values were more pronounced. The highest $OD_{600}$ values were obtained by SRM5(3) (~1.0) and SRM5(1) (~0.9), while the other strains showed $OD_{600}$ values below 0.4 (Figure 3B). The highest μ at this sodium perchlorate concentration was obtained by S1C5(1), SRM5(1), SRM5(2), and SRM5(3), and were statistically higher than the parental strains and S1C5(3) (Figure 4B). At the highest concentration of $NaClO_4$ (90 g $L^{-1}$), the maximum $OD_{600}$ values were lower than in the previous concentrations, with the highest value of only 0.42 being obtained by strain SRM5(2) (Figure 3C). This strain exhibited an µ equivalent to that of the parental strain at this concentration of perchlorate. Among all the strains, SRM5(3) was the only one that had a higher growth rate than its parental strain at this concentration of $NaClO_4$ (Figure 4C).

The results indicate that the mutations caused by Adaptive Laboratory Evolution (ALE) in response to increasing NaCl concentration in the growth medium affected the bacterial growth in sodium perchlorate. However, these mutations only enhanced the growth rate of evolved strains at the intermediate perchlorate concentration of 70 g $L^{-1}$. The strain S1C5(2), which showed the highest NaCl tolerance, did not exhibit higher growth rate than S1C5(W) at any perchlorate concentration. Therefore, despite evidences that ALE with increasing NaCl concentration in the growth medium can improve growth in perchlorate under certain conditions, the mutations associated to tolerance to NaCl and sodium perchlorate seems to be random. Additionally, there is no evidence suggesting that bacteria capable of withstanding higher concentrations of NaCl also possess increased tolerance to perchlorate, even in light of several studies demonstrating the strong performance of halophiles in perchlorate-rich media (Heinz *et al.* 2019; Laye and DasSarma 2018; Matsubara *et al.* 2017; Oren *et al.* 2014). The use of Adaptive Laboratory Evolution (ALE) with NaCl to enhance microbial tolerance to $NaClO_4$ offers a promising and accessible approach for developing astrobiological models. NaCl is a more common, cost-effective, and easier-to-handle alternative to perchlorates, which cannot be sterilized by autoclaving and require filtration due to their thermal instability. This makes NaCl a practical and safer option for increasing bacterial growth and stress tolerance in the context of perchlorate-rich environments.

**Fig. 3** Growth curves of wild and evolved bacteria in sodium perchlorate at 50 g L$^{-1}$ (A), 70 g L$^{-1}$ (B), and 90 g L$^{-1}$ (C).

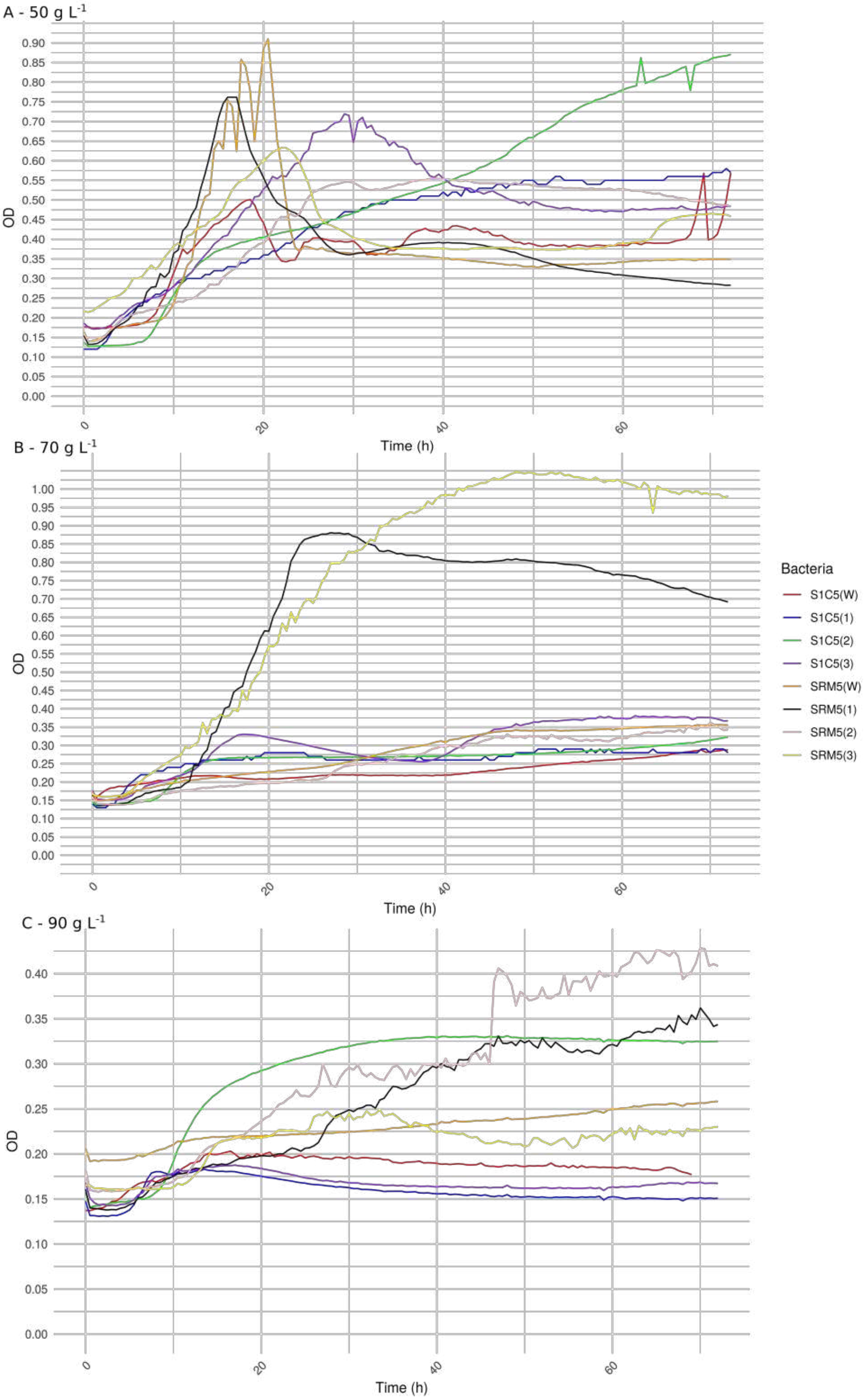
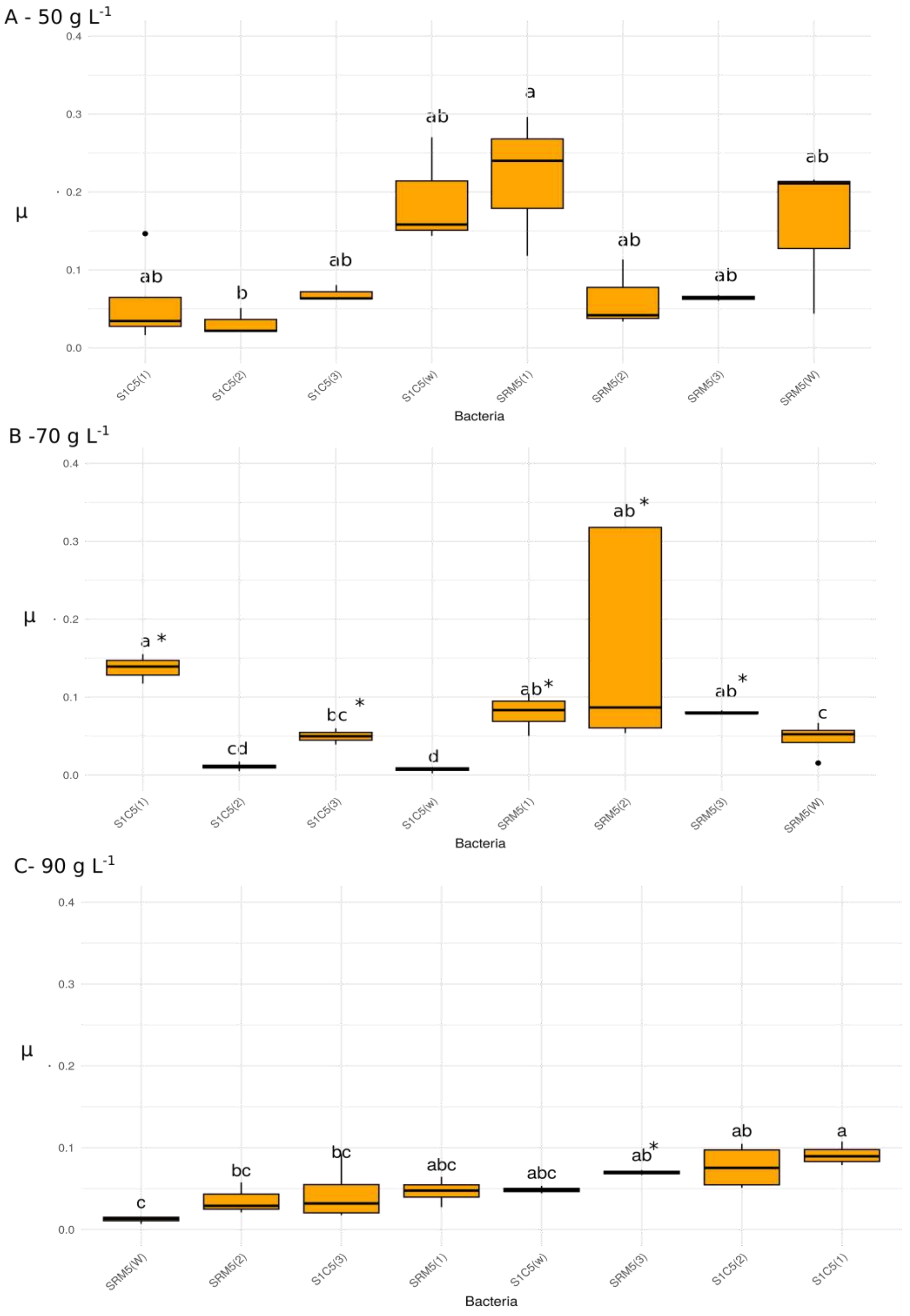


**Fig. 4** Specific growth rate (μ) of wild and evolved strains at 50 g L$^{-1}$ (A), 70 g L$^{-1}$ (B), and 90 g L$^{-1}$ (C) of sodium perchlorate. Strains that have different letters denote significant differences. Strains that exhibited statistically significant differences in μ compared to the parental strain are indicated with an asterisk (*). Results at 50 and 70 g L$^{-1}$ (A and B) were compared using the Kruskal-Walli's test followed by Dunn's test and at 90 g L$^{-1}$ (C) using ANOVA followed by Tukey´s test ($p \leq 0.05$).

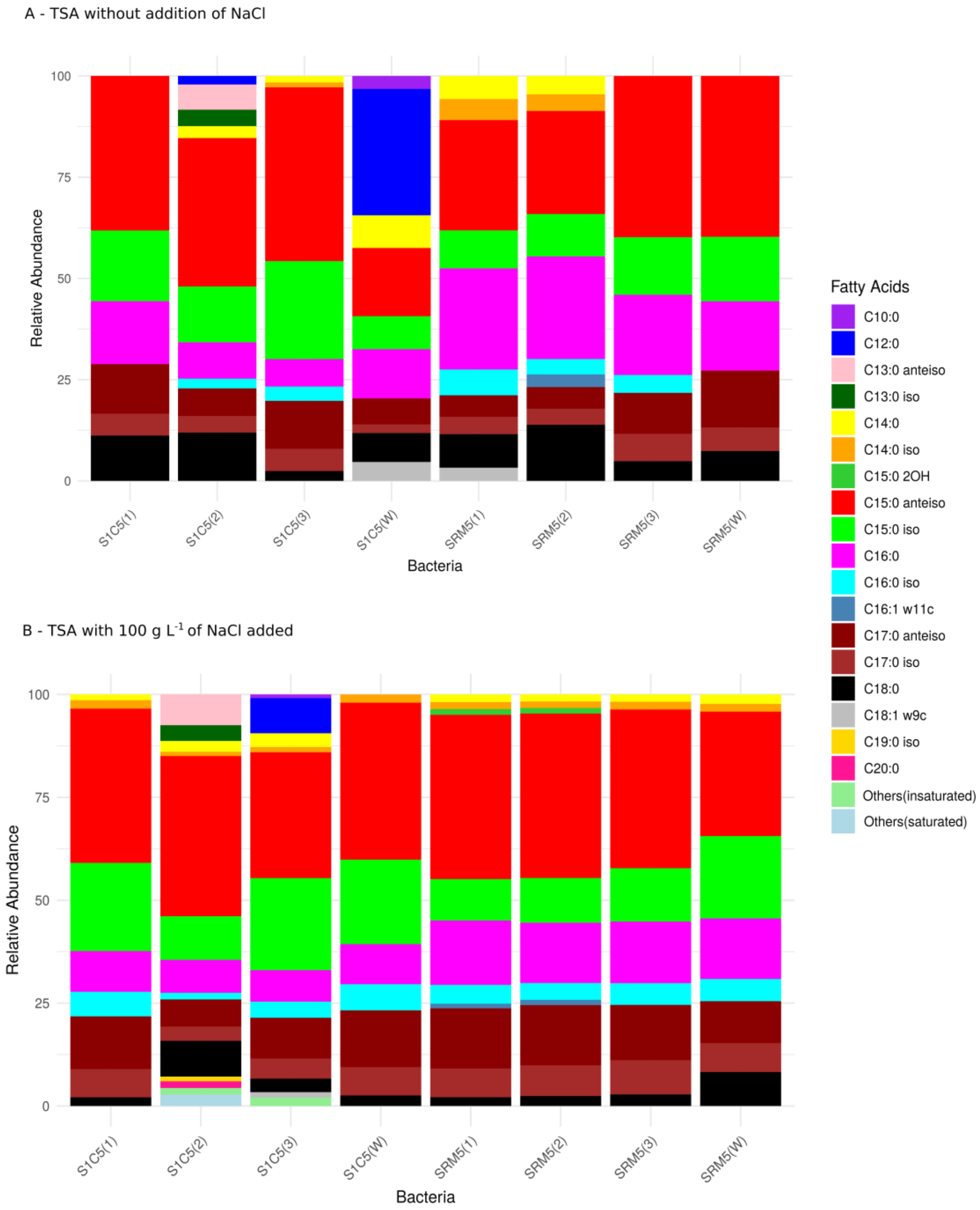


## Effects of ALE on fatty acids profile

Upon examining Figure 5, we observe that certain fatty acids are present in all strains, regardless of the NaCl concentration in the TSA medium used to obtain cell biomass for FAME analysis, differing only in their relative concentrations in the cell. These fatty acids include pentadecanoic acid ($C_{15:0}$ *iso* and *anteiso*), palmitic acid ($C_{16:0}$), heptadecanoic acid ($C_{17:0}$ *iso* and *anteiso*), and stearic acid ($C_{18:0}$). This indicates a predominance of long-chain saturated fatty acids in both halotolerant strains (S1C5 and SRM5). In TSA without NaCl, a notable difference between the parental strain S1C5(W) and the evolved strains is the higher abundance of $C_{12:0}$ (lauric acid) in the parental strain. Only S1C5(2), in addition to the parental strain, exhibited short-chain fatty acids ($<C_{14:0}$). For SRM5, the most significant difference between the parental and evolved strains without salt stress is the presence of $C_{16:0\ iso}$ in the evolved strains, which is absent in the wild strain. The higher abundance of saturated fatty acids may contribute to these organisms' halotolerant status, since the lack of double bonds results in straight chain structures, thereby reducing water loss through osmosis (Mutnuri et al., 2005).

Under saline stress, all strains displayed $C_{16:0\ iso}$ and iso-myristic acid ($C_{14:0\ iso}$). Notably, S1C5(W) at high NaCl lacks lauric acid and was the only strain besides SRM5 that did not produce the non-branched myristic acid ($C_{14:0}$). The evolved strain S1C5(3) produced $C_{10:0}$ and $C_{12:0}$ under salt stress, expressing a fatty acid profile similar to that of S1C5(W) in TSA without NaCl (except for the presence of $C_{14:0\ iso}$ in the former). $C_{12:0}$ was also absent from the fatty acid profile of S1C5(2) at high salinity. Both S1C5(2) and S1C5(3) incorporated unsaturated fatty acids in response to saline stress. This unsaturation enhances membrane fluidity, which may help to prevent cellular lysis caused by water influx (Mutnuri et al., 2005). SRM5(W) and SRM5(3) exhibited similar fatty acid profiles at high NaCl concentrations, differing only in the abundance of some compounds. In contrast, SRM5(1) and SRM5(2) shared similar profiles but differed from other SRM5 strains by the presence of $C_{16:1\ w11c}$, which was already evident in SRM5(2) without added NaCl, and the presence of pentadecanoic acid with two hydroxyl groups ($C_{15:2OH}$). These differences in SRM5 profiles did not align with the kinetic evaluations, as SRM5(1) was more similar to SRM5(W). Additionally, the observed unsaturations, given that all have a methyl-end (w), may contribute to a reduction of the negative charge on membrane ion channels, which helps to repel $Na^+$ and maintain an appropriate concentration of this ion in the cell (De Carvalho *et al.* 2014).

**Fig.5** Relative abundance (%) of fatty acids compounds of evolved and parental strains in TSA without addition of NaCl (A), and TSA with 100 g L$^{-1}$ of NaCl added (B).



## CONCLUSION

The adaptive mechanisms of salt-tolerant microorganisms enable them to thrive not only in environments rich in sodium chloride (NaCl), but also in the presence of other salts. Understanding these mechanisms is essential for scientific advancement and biotechnological applications. In this study, we used adaptive laboratory evolution (ALE) to investigate how this technique influences the growth of evolved strains in NaCl and sodium perchlorate. The results indicated that the evolved microorganisms exhibited greater tolerance to NaCl than their parental strains and demonstrated a shorter generation time when exposed to equal NaCl concentration. Additionally, random mutations induced by ALE positively impacted growth in sodium perchlorate, particularly at 70 g $L^{-1}$, where most of the evolved bacteria showed a higher specific growth rate compared to the wild strains. However, our findings revealed no evidence that a higher specific growth rate in high NaCl concentrations correspond to increased growth rate in perchlorate-rich media. We also observed that the fatty acid profiles of the strains were altered by ALE, although certain fatty acids remained consistent unchanged (except for their proportion in the fatty acid profile), regardless of the strain or stress applied.

This study provides valuable insights into how ALE can enhance NaCl tolerance in halotolerant microorganisms from two underexplored sites in Brazil, providing not only salt-adapted microorganisms but also relevant data about the adaptation process. Moreover, this represents the first investigation into the alterations caused by ALE in response to NaCl stress regarding bacterial fatty acid composition and perchlorate tolerance. This knowledge paves the way for using ALE to develop astrobiological models. Future research will require omics analyses, such as genomic and transcriptomic studies, to gain a deeper understanding of these changes at the molecular level.

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## CONCLUSÃO GERAL

O Brasil é um dos países mais biodiversos do mundo, mas uma parcela muito pequena de sua biodiversidade foi explorada, principalmente a microbiana. O presente trabalho mostrou a importância econômica e científica de se estudar os microrganismos brasileiros, em especial os halotolerantes, visto que o Brasil é um país rico em ambientes salinos que foram pouco explorados. Os microrganismos estudados demonstraram ser aplicáveis na indústria de diferentes formas, uma vez que produzem biossurfactantes, exopolissacarídeos e as enzimas lipase, protease e amilase. Adicionalmente, as bactérias halotolerantes isoladas também podem ser usadas como modelos astrobiológicos, uma vez que, além de tolerarem altas concentrações de NaCl — sal encontrado nas luas de gelo Europa e Enceladus e em Marte —, também são capazes de tolerar e usar perclorato de sódio como único aceptor final de elétrons. Marte é rico em sais de perclorato, e a capacidade de usar esses sais comumente tóxicos como aceptor de elétrons torna viável a habitabilidade neste planeta, que é pobre em oxigênio atmosférico.

Os resultados da evolução adaptativa em laboratório mostraram ser possível usar este método para obter modelos astrobiológicos mais resistentes a perclorato por meio das mutações aleatórias geradas pela exposição sucessiva a altas concentrações de NaCl. Além disso, os resultados cinéticos também mostraram um aumento da tolerância a cloreto de sódio pelas linhagens evoluídas e uma maior dependência deste sal para o crescimento. Adicionalmente, mostramos como a evolução adaptativa em laboratório é capaz de influenciar a resposta fisiológica ao estresse por meio das modificações no perfil de ácidos graxos de membrana. O entendimento das alterações geradas e do potencial dessa técnica para se obter organismos mais resistentes ao estresse salino é importante para futuras aplicações biotecnológicas e astrobiológicas de bactérias halotolerantes, ajudando a identificar alvos para a otimização dessas aplicações.

Futuros estudos devem aprofundar, por meio de estudos ômicos, o entendimento das aplicações biotecnológicas desses microrganismos e das mutações geradas pelo processo de evolução adaptativa em laboratório. Além disso seria interessante o uso desta técnica para desenvolver tolerância a outros fatores estressantes de interesse astrobiológico como luz ultravioleta e temperaturas extremas.