# Understanding *Rhodococcus erythropolis* adaptation to extreme conditions

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Thesis to obtain the Master of Science Degree in

## Biological Engineering

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**October 2018**

# Agradecimentos

Aventura! Aventura acho que é a melhor palavra que descreve estes 6 anos que findam com esta última etapa, e que tanto me fizeram crescer e ser a pessoa que sou hoje. Muitas foram as pessoas que me acompanharam neste meu percurso com altos e baixos, e como tal, merecem ser relembradas e homenageadas por tudo.

Em primeiro lugar, tenho de agradecer aos meus pais Vitor e Lina e ao meu irmão João, por todo o carinho, apoio, ajuda, incentivo, força, otimismo e compreensão ao longo destes anos, sem nunca duvidarem de mim. Sem vocês, nada disto teria sido possível! A pessoa que sou hoje, devo-o a vocês!

À minha orientadora, Doutora Carla de Carvalho, por todo o apoio, paciência, dedicação e disponibilidade manifestados ao longo destes meses de trabalho. Foi sem qualquer dúvida, uma excelente experiência e um privilégio trabalhar consigo! Espero ter estado à altura!

Ao Carlos Rodrigues, por toda a paciência, disponibilidade e ajuda prestada ao longo do trabalho, pela preocupação em saber se tudo estava a correr bem e o mais importante pela integração nesta pequena grande família.

Ao Ricardo Pereira, colega de curso, não só pelos esclarecimentos no funcionamento de alguns equipamentos e procura de reagentes, mas também pelo apoio, conselhos e disponibilidade prestada ao longo destes meses.

Ao Filipe Carvalho, por todos os sermões e repreensões, mas também pelo apoio e interesse em ajudar e tentar saber mais sobre uma área que não está relacionada com o teu trabalho. Eu prometo que no final te ofereço o famoso travesseiro de Sintra, mas só se me trouxeres um Viriato de Viseu!

À Dª. Rosa por ser a nossa “mãe”, por ter sempre uma palavra reconfortante para nos dar e por ser, para além de um excelente profissional, uma pessoa maravilhosa com um coração gigante.

Aos meus colegas de laboratório/mestrado, Daniela Silva, Catarina Pereira, Maryna Bondar, Beatriz Silva, Ricardo Barreiros e Filipe Matias, por terem tornado estes meses de trabalho numa excelente etapa marcada pela boa disposição, companheirismo e incentivo. Os dias no laboratório passavam sempre mais rápido na vossa companhia!

À Daniela Silva e Catarina Pereira, quero ainda agradecer por me terem ajudado sempre que vos pedia, por aturarem o meu mau humor sempre que alguma coisa corria mal e o principal, por terem sempre uma palavra certa para me dizer quando entrava em stress desnecessariamente. À Daniela agradeço também por me ficares a fazer companhia no laboratório, quando já tinhas terminado o teu trabalho e todos já se tinham ido embora.

À minha melhor amiga Inês Gonçalves, a minha mana do coração, por teres estado presente nos bons e maus momentos, por todo o apoio incondicional, mesmo quando o meu ultra mau humor e stress recaíam sobre ti, pela motivação e incentivo e por mesmo não percebendo muito do assunto, teres

estado sempre interessada em saber como estavam as minhas "filhas" e curiosa em relação a tudo o que fazia! Agradeço também ao meu “mano” Paulo Gonçalves pelas ajudas e conselhos ao longo destes anos!

À Adriana Rijo, a nossa pseudo Checa e boss de laboratório, por te teres revelado uma excelente pessoa, uma amiga sempre presente e por estares sempre preocupada e interessada em saber como estavam a correr as experiências, mesmo andado sempre cheia de trabalho.

Aos meus amigos Iselianos, Rafaela Peralta, Inês Santos, André Pinto, Francisco Santos, Cláudia Carvalho, Luís Marques e Cláudia Ferreira por me terem acompanhado nesta aventura, por todos os bons momentos e por todos os ensinamentos que me transmitiram! Vocês são a prova de que mesmo que cada um siga um caminho diferente e estejamos meses sem estarmos juntos, é possível manter-se uma boa amizade.

Aos meus amigos Tecnianos, Sara Figueira, Solange Bolas, Helena Herrera, Rita Almeida, Margarida Afonso, João Morais, Lisa Azevedo e João Santana pela integração na vossa turma, pelo companheirismo, pelo apoio e ajuda. Foram um apoio muito importante nesta última etapa!

A todos os meus outros amigos, colegas e professores por terem de alguma forma enriquecido esta minha aventura!

Todos vocês tornaram este percurso muito mais fácil e por isso, irei estar-vos eternamente grata!!

Por último, gostaria de agradecer à Fundação para a Ciência e a Tecnologia pelo financiamento parcial destes estudos através do projecto exploratório IF/01203/2013/CP1163/CT0002.

# Abstract

Bacterial cells are exposed to several environmental conditions, and they use various adaptive mechanisms to survive. The genus *Rhodococcus* is important in biotechnology and industry due to its adaptability to diverse conditions and metabolic diversity. Several strains have been studied for the understanding of their metabolic pathways and the adaptive mechanisms.

The main objective of this work was to study the role of lipids of *Rhodococcus erythropolis* DCL14 during adaptation to dehydration. Analysis of the fatty acid profile revealed that, regardless of the cell age at the time of harvest and the composition of the growth medium, the bacterium when grown in planktonic state, increased the degree of saturation of the fatty acids of the cell membrane, suggesting that of the cells reduced membrane permeability as response to dehydration. However, when grown as biofilm, the cells did the opposite, indicating that the growth mode has stronger impact in cell adaptation. In addition to studying the changes in fatty acid composition, the effect of dehydration on the net surface charge of the cells was also analysed, and it was verified that with increasing dehydration time the cells became less negative.

To assess if the presence of storage lipids could help the survival of the cells under stressful conditions, the influence of these lipids on the tolerance of the cells to dehydration and to the antibiotic vancomycin was studied. Results following dehydration indicated an increase in the degree of saturation of the fatty acids of the cell membrane, while results following vancomycin exposure indicated a biphasic killing kinetics, indicating the presence of persister or resistant cells, depending on the source of carbon used.



**Keywords:** *Rhodococcus*, dehydration, adaptation, fatty acids, storage lipids, vancomycin tolerance

# Resumo

As células bactérias encontram-se expostas a diversas condições ambientais, e usam diversos mecanismos adaptativos para sobreviver. O género *Rhodococcus,* é importante em biotecnologia e em alguns sectores da indústria devido à sua adaptabilidade a diversas condições e diversidade metabólica. As via metabólicas e os mecanismos de adaptação de diversas estirpes têm sido estudados.

O principal objetivo deste trabalho foi estudar o papel dos lípidos de *Rhodococcus erythropolis* DCL14 durante a adaptação à desidratação. A análise do perfil de ácidos gordos relevou que, independentemente da idade das células no momento de recolha e da composição do meio de crescimento, a bactérias quando crescida em estado planctónico, aumentava o grau de saturação dos ácidos gordos da membrana, sugerindo que as células reduziram a permeabilidade da membrana como resposta à desidratação. No entanto, quando crescidas como biofilme, as células fizeram o oposto, indicando que o modo de crescimento tem um impacto significativo na adaptação celular. Para além de se estudarem quais as alterações na composição de ácidos gordos da membrana celular, o efeito da desidratação na carga superficial das células foi também analisado, verificando-se que as células se tornaram menos negativas com o aumento do tempo de desidratação.

Para avaliar se a presença de lípidos de reserva poderia ajudar na sobrevivência das células sob condições de stress, foi estudada a influência destes lípidos na tolerância das células à desidratação e ao antibiótico vancomicina. Os resultados após desidratação indicaram um aumento no grau de saturação dos ácidos gordos da membrana, enquanto que os resultados após exposição à vancomicina indicaram uma cinética de morte bifásica, indicando a presença de células persistentes ou resistentes, dependendo da fonte de carbono utilizada.



**Palavras-chave:** *Rhodococcus*, desidratação, adaptação, ácidos gordos, lípidos de reserva, tolerância a vancomicina

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# List of Abbreviation

| | |
|---|---|
| **10MBFAs** | 10-methyl branched fatty acids |
| **ATP** | Adenosine triphosphate |
| **BCFAs** | Saturated *iso*- and *anteiso*-methyl branched fatty acids |
| **BEHP** | Bis(2ethylhexyl) phthalate |
| **BTEX** | Benzene-Toluene-Ethylbenzene-Xylene |
| ***ca.*** | *circa* or approximately |
| **CDW** | Cellular dry weight |
| **CFU** | Colony forming unit |
| **CL** | Cardiolipin |
| **DBTs** | Dibenzothiophenes |
| **DMA** | Dimethylacetals fatty acids |
| **DMF** | Dimethylformamide |
| **eDNA** | Extracellular deoxyribonucleic acid |
| **Dsat** | Degree of saturation |
| ***e.g.*** | *exempli gratia* or for exemple |
| **EPS** | Exopolymeric substances |
| **FA** | Fatty acid |
| **FAMEs** | Fatty acid methyl esters |
| **FID** | Flame ionization detector |
| **GC** | Gas chromatograph |
| **$K^+$** | Potassium ion |
| **LAM** | Lipoarabinomannan |
| **MA** | Mycolic acids |
| **MH** | Mueller-Hinton media |
| **MIC** | Minimum inhibitory concentration |
| **MM** | Mineral medium |
| **MUFAs** | Monounsaturated fatty acids |
| **OD** | Optical density |
| **PAHs** | Polycyclic aromatic hydrocarbons |
| **PCBs** | Polychlorinated biphenyls |
| **PE** | Phosphatidylethanolamine |
| **PG** | Phosphatidylglycerol |
| **PHA** | Polyhydroxyalkanoates |
| **PI** | Phosphatidylinositol |
| **PIM** | Phosphatidylinositol mannoside |
| **PLFA** | Phospholipid fatty acids |
| **PMF** | Proton motive force |
| **PUFAs** | Polyunsaturated fatty acids |
| **rpm** | Rotation per minute |
| **SCBFAs** | Saturated cyclopropyl-branched fatty acids |
| **SSFAs** | Saturated straight chain fatty acids |
| **TAGs** | Triacylglycerols |
| **TSA** | Tryptic soy broth |

# 1. Introduction

During the last decades it has become clear that organisms can be found in all type of environments, including under extreme conditions. These extreme conditions could be physical (*e.g.* temperature, pressure or radiation) or/and geochemical (*e.g.* desiccation, salinity or pH). The organisms that inhabit environments in such conditions are known as extremophiles (Rothschild and Mancinelli 2001). The interest of scientists on extremophiles results from their biochemical pathways and enzymes that are stable and active under extreme conditions, having an important role in biotechnology, principally now with the increasing industrial demands for biocatalysts that can work at harsh industrial process conditions (Morozkina, Slutskaya *et al.* 2010).

Extremophiles organisms are classified according to conditions in which they grow **(Table 1)**, and the enzymes obtained from these organisms could theoretically be applicable under similar condition, offering many opportunities for a variety of applications (Morozkina, Slutskaya *et al.* 2010; Kumar, L., Awasthi *et al.* 2011; de Carvalho 2017). This group of organisms can be divided into two categories: extremophilic, which are organisms that require one or more extreme conditions to grow; or extremotolerant which are organisms that can tolerate one or more extreme conditions, but grow optimally under moderate (Rampelotto 2013).

**Table 1 –** Classification and example of some extremophiles (adapted from Rothschild and Mancinelli 2001; Rampelotto 2013).

| Environmental Parameter | Type | Definition |
|---|---|---|
| **Temperature** | Hyperthermophile | $T_{op} > 80$ ºC |
| | Thermophile | $60 < T_{op} < 80$ ºC |
| | Psychrophile | $T_{op} < 15$ ºC |
| **pH** | Alkaliphile | $pH_{op} > 8.5$ |
| | Acidophile | $pH_{op} < 3$ |
| **Pressure** | Barophile/Piezophiles | Grow better under pressure |
| **Salinity** | Halophile | Require NaCl for growth (NaCl > 3%) |
| **Desiccation** | Xerophiles | Grow at low water availability |
| **Radiation** | Radioresistant | Grow at high levels of ionizing radiation (*e.g.* UV) |

In addition to the classifications shown in the **Table 1**, some organisms live in environments that have more than one physicochemical parameter reaching extreme values, and for this they are considered polyextremophiles (Rothschild and Mancinelli 2001). However, the parameters mentioned above are not the only ones considered extreme, because there are some organisms that can grow in places contaminated with *e.g.* heavy metals or organic solvents at concentrations higher than those supported by the majority of living cells (Isken and de Bont 1998; Rampelotto 2013).

Extremophilic organisms have the ability to inhabit extreme environments due to their particular characteristics, such as a unique composition of the cellular membrane, various bioactive metabolites, thermostable membrane proteins and enzymes active under unusual conditions, some with a high turnover rate (Rothschild and Mancinelli 2001). However, the industrial application of these organisms has drawbacks, such as the longer generation time and lower biomass yields than mesophilic

organisms. Thus, to overcome these problems it became standard to clone the specific genes of a desired enzyme into a mesophilic host, leading to an overproduction (de Carvalho 2011;2017). However, it is possible to use extremotolerant organisms that can survive under extreme conditions, even if they have higher growth rates when grown under moderate conditions. Adapted mesophilic bacteria to extreme environments, with biocatalytic abilities that are desired in industry, could improve the production of compounds with commercial interest or its use in bioremediation (de Carvalho and da Fonseca 2005b; de Carvalho 2012).

The group of actinomycetes bacteria have metabolic diversity and have been isolated from several habitats. This demonstrate that actinomycetes, have characteristics that allows them to adapt and live under diverse conditions. Actinomycetes are a group of gram-positive bacteria, belonging to the phylum Actinobacteria*,* that are characterized as aerobic and filamentous bacteria with a high G+C content, but also as a free-living organism that are present in all types of ecosystems (mainly in the soil) and as bacteria with a high metabolic diversity (Barka, Vatsa *et al.* 2016). This group comprises bacteria able to cause infections such as *Mycobacterium tuberculosis* and *Rhodococcus equi,* but also strains able to produce products with industrial interest such as antibiotics, and to decompose recalcitrant compounds (de Carvalho and da Fonseca 2005b; de Carvalho, Costa *et al.* 2014; Sharma, Dangi *et al.* 2014). Their metabolites are used in a wide range of applications, from agriculture and environmental remediation, to the production of antibiotics. For this reason, it that has become important to explore remote areas such as the marine environment, extreme arid and salty areas and glaciers, in order to find new strains that produce novel products to boost the biotechnology industry (Tiwari and Gupta 2013).

Of the total microbial bioactive secondary metabolites produced, about 45% are produced by actinomycetes, of which 34% are produced by *Streptomyces* and the remaining 11% by non-*Streptomyces* (Mahajan and Balachandran 2012). Two-third of the compounds produced by actinomycetes are antibiotics, including those that are in clinical use, while one-third of them are a wide range of compounds with medical relevance such as antifungal, antiprotozoal, antiviral, anticholesterol, anti-cancer drug and immunosuppressive agents (Adegboye and Babalola 2013). About 75% of the existing antibacterial compounds are produced by actinomycetes, exhibiting a plethora of mechanisms of action with a high potency against numerous gram-positive and gram-negative bacteria.

Besides the production of antibiotics, which have an important role in the combat of infectious diseases, actinomycetes have a great potential for other applications **(Figure 1)** such as bioremediation processes (de Carvalho and da Fonseca 2005b; de Carvalho 2012; de Carvalho, Costa *et al.* 2014; Sharma, Dangi *et al.* 2014). In bioremediation processes there are several reports that show the great potential of actinomycetes, such as the use of six strains to clean up soil contaminated with crude oil (Burghal, Al-Mudaffarand *et al.* 2015). In another study performed by Polti *et al.,* five *Streptomyces* strains were used to remediate soil co-contaminated with Cr-(VI) and lindane (Polti, Aparicio *et al.* 2014). Besides that, the genus *Rhodococcus*, has its potential for bioremediation shown in several studies, such as the use of *R. pyridinivorans* NT2 strain in nitrotoluene-contaminated soils (Kundu, Hazra *et al.* 2016) or the use of *R. wratislaviensis* strain 9 to degrade *p*-nitrophenol (PNP) in groundwater contaminated (Subashchandrabose, Venkateswarlu *et al.* 2018); more examples in section 1.1.1.).

Within the wide range of bioremediation studies made with the genus *Rhodococcus,* de Carvalho and co-workers focused the studies in the biocatalytic and bioremediation potential of the species *R. erythropolis,* showing it in several studies (de Carvalho and da Fonseca 2005a; de Carvalho, Parreno-Marchante *et al.* 2005; de Carvalho, Fatal *et al.* 2007; de Carvalho, Wick *et al.* 2009; de Carvalho, Marques *et al.* 2014), including a study describing the capability of this species to grow under diverse conditions, that portrays some of the conditions where these bacteria can be find in the environment (de Carvalho 2012). Thus, the study of the influence of other environmental conditions on the growth of these microorganisms and the understanding of how these can be used in bioremediation of certain contaminated areas is very important and could lead to new discoveries and applications in the biotechnology industry.

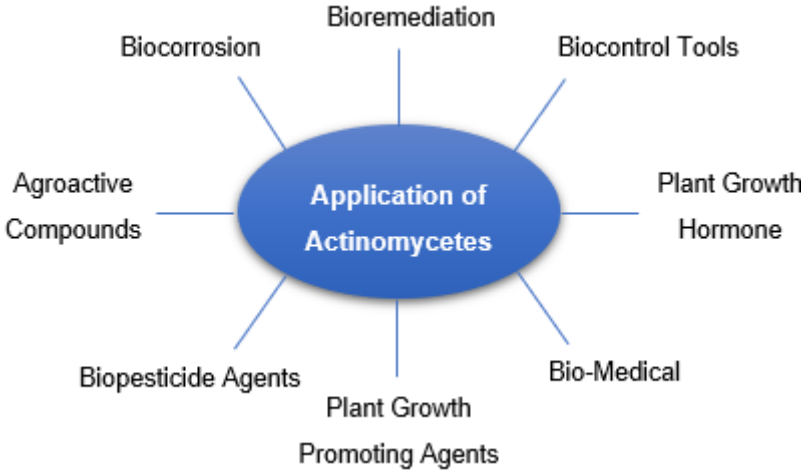


**Figure 1 –** Fields of application of Actinomycetes (adapted from Sharma, Dangi *et al.* 2014).

## 1.1. The genus *Rhodococcus*

The genus name *Rhodococcus* was first proposed in 1891 by Zopf (Zopf 1891) to include *Micrococcus erythromyxa* and *M. rhodochrous*, two red bacteria described by Overbeck (Overbeck 1891) in the same year. In 1906, Winslow and Rogers and in 1907 Molisch, also proposed the genus *Rhodococcus* for other red bacteria (Winslow and Rogers 1906). During the first four editions of Bergey's Manual of Determinative Bacteriology, this genus was preserved with a *R. rhodochrous* as a type strain, but in the fifth and sixth editions some strains, including *R. rhodochrous*, were revaluated and incorporated in the genus *Micrococcus.* A lack of appropriate characteristics to describe these strains still persist in the seventh edition, and for that there was no consensus on which genus these *rhodochrous* strains belonged to (Gűrtler and Seviour 2010). Initially, based mainly on similarities in colony morphologies and acid fastness of some cultures, Gordon placed all these *rhodochrous* strains into the genus *Mycobacterium* as *M. rhodochrous* (Gordon 1966). However, in the eighth edition of Bergey's Manual of Determinative Bacteriology, these *rhodochrous* strains were removed from the genus *Mycobacterium* and incorporated tentatively to a number of *Nocardia* species (McClung 1974). Bousefield and Goodfellow, in 1976, considered that *rhodochrous* strains might be classified as *Mycobacterium rhodochrous*, reclassified in an alternative established genus or considered as a new genus (Bousfield and Goodfellow 1976). However, it was in only in 1977 that Goodfellow and Alderson

reintroduced the genus name *Rhodococcus* to include the *rhodochrous* strains that resembled but did not belong to the established genera *Corynebacterium, Mycobacterium* and *Nocardia* (CMN group) (Goodfellow and Alderson 1977).

The bacteria of the genus *Rhodococcus*, in terms of taxonomy, belong to the phylum *Actinobacteria*, order *Actinomycetales,* suborder *Coryneabacterineae* and family *Nocardiaceae* (Goodfellow 1989a; Gűrtler and Seviour 2010). *Rhodococcus* bacteria are described as gram-positive, aerobic chemo-organotrophs, non-motile, mycolic acid-containing, nocardioform and non-sporulating, which have a high G+C content (63-73%). *Rhodococcus* cells have the distinctive ability to synthesize highly branched mycolic acids with range from 30 to 54 carbons atoms and more than 4 double bonds, reason why they belong to the mycolata taxon (Bell, Philp *et al.* 1998; Goodfellow 2012). *Rhodococcus* colonies morphologically can be mucoid, smooth or rough, measuring 0.25-2 mm in diameter (depending on cell age) and in terms of colour can be coral, orange, pink, red or yellow. Most of strains have a range of temperature of cultivation from 15ºC to 40ºC (Gűrtler and Seviour 2010; Goodfellow 2012). Furthermore, rhodococci contain an oxidative metabolism enabling them to use different organic compounds and pollutants as energy source and sole carbon, such as hydrocarbons, steroids, lignin, and chlorinated phenolics, but also to produce diverse compounds and molecules with industrial interesting such as carotenoid pigments, bio-flocculation agents, biosurfactants, amides and polymers (Finnerty 1992; Bell, Philp *et al.* 1998).

*Rhodococcus* strains have been isolated from samples collected in a wide diversity of sources such as soils, coastal sediments, rocks, herbivorous dung, boreholes and groundwater (Bell, Philp *et al.* 1998) but also some from environments with extreme conditions like the semiarid Patagonia and the Andean Puna in South and North of Argentina (Ordoñez, Flores *et al.* 2009; Silva, Grossi *et al.* 2010; Urbano, Albarracín *et al.* 2013).

Nowadays, there are 63 species belonging to this genus (Euzéby 1997) and some members show pathogenicity for humans, animals and plants, such as *R. fascians* and *R. equi*. Based in phylogenetic data from 16S rRNA sequences, rhodococci are divided in three taxa in gene tree: *R. erythropolis, R. rhodochrous* and *R. equi* subclasses (Goodfellow 2012).

### 1.1.1. Importance of *Rhodococcus* in Bioremediation and Biodegradation

The deliberate or accidental introduction of pollutants into environment has become one of the most urgent global problems with noteworthy threats to human health and natural biodiversity. Environmental contaminants are the result *e.g.* of industrial leaks and spills, improper application of pesticides, oil spillage and oily waste deposits. These contaminants may be organic or inorganic compounds like heavy metal, alkanes, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), chlorobenzenes, chlorophenols or toxic chemical fertilizers (Alexander 1999).

The treatment of soil contaminated with organic compounds is difficult for several reasons, such as the sorption of contaminants to the soil matrix but also their low water solubility and limited rate of mass transfer. Besides, remoteness and low accessibility to the contaminated sites, adverse environmental conditions, elevated levels of pollution and a huge amount of contaminated material to be treated, difficult the remediation activities (Alexander 1999). Remediation activities are a costly

method and produce hazardous products, so bioremediation is an alternative. Bioremediation is a technology based on use of microorganisms to degrade toxic compounds into nonhazardous substances (Colleran 1997; Tyagi, da Fonseca *et al.* 2011). This method of biological remediation is considered nondestructive, low cost and environmentally safe (Kuyukina and Ivshina 2010), although not all microorganisms can be used due to a limited tolerance to the cytotoxicity of the pollutants and lack of appropriate metabolic routes (Singleton 1994). Thus, it is important to select a strain able to tolerate the toxicity of the substrate and to convert it into less toxic compounds.

Rhodococci cells have a metabolic diversity that makes possible several biocatalytic reactions and degradations. For this reason, *Rhodococcus* genus is a favourable candidate to use in bioremediation. *Rhodococcus* strains are resistant to a vast number of recalcitrant compounds and have the ability to degrade and/or convert a wide range of hydrophobic natural compounds and xenobiotics, including short-chain and long-chain alkanes, nitroaromatics (Larkin, Kulakov *et al.* 2005), aliphatic and aromatic hydrocarbons (*e.g.* BTEX-Benzene, Toluene, Ethylbenzene and Xylene) (Martinkova, Uhnakova *et al.* 2009), alcohols, phenolic compounds, ethers, nitriles, pesticides (Kuyukina and Ivshina 2010), PAHs, PCBs and dibenzothiophenes (DBTs) (van der Geize and Dijkhuizen 2004). This metabolic diversity and versatility are related to the presence and mobilization of large linear plasmids and with the presence of multiple homologues enzymes in catabolic pathways (de Carvalho and da Fonseca 2005b).

Reports on the metabolic versatility of *Rhodococcus* species show the ability of several species to degrade toxic compounds: *R. rhodochrous* degrade PCBs (Boyle, Silvin *et al.* 1992); *R. corallinus* degrade atrazine and s-triazine (Larkin, Kulakov *et al.* 2005; Arnold, Hickey *et al.* 2009); *R. aetherivorans* degrade t-butylether; *R. gordoniae* degrade phenol; *R. pyridinivorans* degrade pyridine (Goodfellow 2012); *R.jostii* RHA1 degrade and transform PCB and other aromatic compounds (Hernández, M.A., Mohn *et al.* 2008); and *R. ruber*, *R. erythropolis* and *R. opacus* degrade crude-oil (Ivshina, Kuyukina *et al.* 1998). Relatively to the strain *R. erythropolis* DCL14, reports show its ability to degrade a wide range of toxic compounds, such n-alkanes, alcohols, terpenes (de Carvalho, Parreno-Marchante *et al.* 2005; de Carvalho, Wick *et al.* 2009), aromatic compounds (de Carvalho, Fatal *et al.* 2007), fuel oil and motor oils (de Carvalho and da Fonseca 2005b).

Besides the biodegradative abilities, *Rhodococcus* strains are able to produce biosurfactants (microbial surface-active agents) in response to the presence of hydrocarbons in soil and water (Lang and Philp 1998). Biosurfactants produced in rhodococci are predominantly glycolipids (containing trehalose as the carbohydrate) (Lang and Philp 1998), with tensoactive properties and a threefold relevance in bioremediation. Firstly, cellular surfactants in rhodococci promote the adhesion of the cells to the hydrophobic phase in two-phase systems. Secondly, they decrease the interfacial tension between organic-aqueous, allowing an easier entry of the hydrophobic compounds into the microbial cells and, thirdly, they increase the surface area for microbial degradation due to a dispersion of the hydrophobic compounds (Bell, Philp *et al.* 1998). Their particular characteristics are used in several industries (*e.g.* pharmaceutical, food, cosmetic) (Banat, Makkar *et al.* 2000) as emulsifiers, wetting and foaming agents, detergents, flocculating agents or functional food ingredients (Singh, Van Hamme *et al.* 2007).

### 1.1.2. Cell wall and cell membrane composition

The cell envelope of a Gram-positive bacterium is constituted for cell membrane, cell wall and other structures that surround and protect the cytoplasm. The cell envelope creates a protective and impermeable barrier, playing an important role in bacterial growth and survival in environments with adverse conditions (Silhavy, Kahne *et al.* 2010). Besides functioning as a protective barrier, the cell envelope also regulates the cell shape, mediates cell adhesion to surfaces and other cells, participate in a selective passage of nutrients and waste products, and gives support to cytoplasmic membrane against high osmotic pressure (Lambert 2002).

The cell envelope of rhodococci is highly organized, in comparison with other gram-positive, and is constituted by a mycolyl-peptidoglycan-arabinogalactan complex. Some of the constituents of this complex are 2-alkyl branched, 3-hydroxy long-chain fatty acid, known as mycolic acids (MA). MA are characteristics for the mycolata taxon and are found in the following genera: *Corynebacterium, Dietzia, Gordonia, Mycobacterium, Nocardia, Rhodococcus, Skermania* and *Tsukamurella*. These lipids represent more than 40% of the total cell wall and vary in size and complexity depending on the genera (Sutcliffe, I.C. 1998). Those of the genus *Mycobacterium* are the most complex, with a chain of 60-90 carbons and a greater diversity of meromycolate chain functional group such as methoxy- and keto-modifications, and cyclopropane, while those of the genus *Corynebacterium* are the smallest with a chain of 22-38 carbons (Sutcliffe, I.C. 1998; Sutcliffe, I.C, Brown *et al.* 2010). In comparison to the other genera of the mycolata taxon, MA of *Rhodococcus* have an intermediate size (30 to 54 carbons) (Sutcliffe, I.C. 1998). The length of the meromycolate chain compared with the length of the alkyl side branch is an important feature in the structure of MA. Typically, the meromycolate chain contains 20-42 carbons with up to four double bonds, while the alkyl chain is shorter with 10-16 carbons (Stratton, Brooks *et al.* 1999). Since the length, structure and complexity of MA vary between genera and strains, they can be used as a marker for taxonomic classification (Kellogg, Bankert *et al.* 2001).

MA represent the basis of an outer lipid permeability layer and they are covalently linked to the cell wall polysaccharide arabinogalactan, which is a biopolymer composed of arabinose and galactose monosaccharides. This biopolymer there is covalently linked to peptidoglycan (chemotype IV). The presence of MA confers hydrophobic character to *Rhodococcus* cells, helping the degradation of hydrophobic compounds (Goodfellow 2012).

Rhodococcal peptidoglycan consists in *N*-acetylglucosamine, *N*-glylcolylmuramic acid, *D*- and *L*-alanine, and *D*-glutamic acid with *meso*-diaminopimelic acid, which is directly cross-linked in positions three and four of tetrapeptides - A1 γ type (Lechevalier and Lechevalier 1970). The peptidoglycan is linked to the galactan domain of arabinogalactan through a well conserved linker unit (LU) of *L*-rhamnose-*D*-*N*-acetylglucosamine phosphate. Each domain of galactan typically carry three arabinan domains, which have in pentarabinosyl branched termini four MA **(Figure 2)** (Lechevalier and Lechevalier 1970; Sutcliffe, I.C, Brown *et al.* 2010). Sutcliffe referred that there are some variations in the linkage types within the galactans domain, but also a diversity in terminal motif of arabinan domain which influences the number of MA that each terminal can carry in the different genera studied, including the genus *Rhodococcus* (Sutcliffe, I.C. 1998).

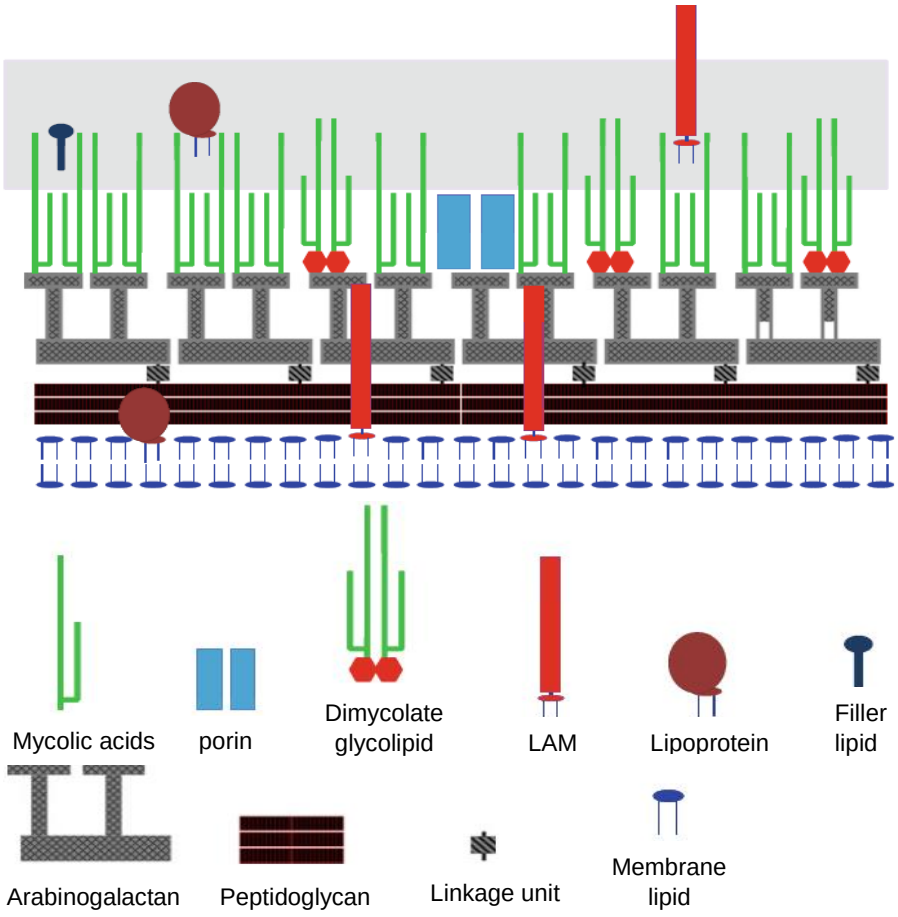


**Figure 2** – Cell wall structure of mycolata taxon (Biology of *Rhodococcus* by Héctor M. Alvarez, Copyright © 2010, Reprinted with permission from Spring Nature Customer Service Centre GmbH: Springer)

The first studies about the organization and composition of the rhodococci cell wall showed that MA made 30% of the dry weight of the cell wall skeleton and were arranged in a parallel manner to each other, and in a perpendicularly manner to the cell wall. Recent studies, with *R. erythropolis* and *R. opacus,* suggested that hydrocarbon-like compounds make 40% of the dry weight of the cell wall, where 20% of this weight is attributed to the heteropolymer arabinogalactan (Sutcliffe, I.C. 1998).

As illustrated in **Figure 2**, besides the mycolyl-peptidoglycan-arabinogalactan complex there are other compounds non-covalently associated that make the cell envelope, like as channel forming porins, lipoglycans, lipids and lipoproteins.

Channel forming porins are non-specific protein channels, embedded in the cell wall and membrane, that allow the passage of molecules through the membrane. Due to the lipid permeability barrier, the hydrophilic molecules are unable to cross the cell wall and, they pass through porins (Achouak, Heulin *et al.* 2001). The presence of porins in rhodococci have been identified in some species, such as *R. corynebacteroides* (Rieß and Benz 2000), *R. erythropolis* (Lichtinger, Reiss *et al.* 2000), and in *R. equi* (Rieβ, Elflein *et al.* 2003).

Lipoglycans are polysaccharides covalently anchored to the membrane, which is characteristic of the Actinobacteria cell wall (Sutcliffe, I.C. 1995). These polysaccharides belong to the lipoarabinomannan (LAM) family (Nigou, Gilleron *et al.* 2003), and, like the arabinogalactan, have a core

structure characterized by the presence of a membrane anchor base on phosphatidylinositolmannoside, which has a varying structure depending on the species and is strain-specific. The mannan core can also carry arabinose or arabinan branches, besides mannose side chains, and for that, there are a variety of substituent motifs (Nigou, Gilleron *et al.* 2003).

Although lipoarabinomannan and structurally related lipomannan lipoglycans are characteristic of the genus *Mycobacterium*, is possible to find similar lipoglycans in other actinomycetes, such as the genus *Rhodococcus*. The first report of lipoglycans in the genus *Rhodococcus* was in 1996 by Flaherty *et al.* in 1996 for the strain *Rhodococcus rhodnii* N445, evidencing that these lipoglycans are widely distributed by the mycolic acid-containing actinomycetes. However, these studies failed to show that such lipoglycans had biologic activities similar to mycobacterial LAMs (Flaherty, Minnikin *et al.* 1996). These *Rhodococcus* lipoglycans show typical components of LAM but has been found variation in monosaccharide composition. This variation in monosaccharide composition created a distinct subfamily, the truncated LAMs (Garton, Gilleron *et al.* 2002; Gilleron, Garton *et al.* 2005). The first known example of a truncated LAM was found in *R. equi,* wherein the structure of LAM-like lipoglycan (ReqLAM), beyond the typical phosphatidylinositolmannoside-anchored core, has in mannose branches a single *t*-arabinofuranose residue (Garton, Gilleron *et al.* 2002). Likewise, in the structure of LAM-like lipoglycan of *R. ruber* (RruLAM) the lipomannan core is directly substituted with *t*-arabinofuranose residues (Gibson, Gilleron *et al.* 2003).

The cytoplasmatic membrane of rhodococci has a structure homologous to the structure of other bacteria, constituted with polar lipids, more specifically glycerophospholipids. The phospholipids pattern consists in cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylinositol mannosides (PIMs) (Finnerty 1992). These polar lipids are composed by fatty acids (FAs), with chains containing saturated straight chain (SSFAs), 10-methyl branched (10MBFAs) and monounsaturated (MUFAs) fatty acids. The main FAs are hexadecanoic (or palmitic acid – C16:0), octadecenoic (or oleic acid – C18:1) and 10-methlyoctadecanoic (or tuberculostearic – 10MeC18:0) acids (Barton, Goodfellow *et al.* 1989). Besides polar lipids, the cell wall of *Rhodococcus* haver other lipids denominated as “free lipids”, because there are lipids non-covalently linked to the cell envelope. These “free lipids” are trehalose dimycolates, glycosyl monomycolates and peptidolipids, that together with the MA form an asymmetric bilayer (Sutcliffe, I.C. 1997).

#### 1.1.2.1. Adaptive mechanism at the membrane level in *Rhodococcus*

Biological membranes have as function the maintenance of cell integrity, ensuring a separation barrier between the intra- and extracellular space, but also the exchange of substrates and energy. The dynamic and structural characteristics of the membranes may change due to changes in environmental conditions, which may be related with physicochemical parameters such as temperature, pH, pressure, but also with the presence of toxic compounds. These environmental stresses promote changes in membrane fluidity, permeability and structure, which consequently affect the functions and mobility of membrane proteins, the diffusion of nutrients and the appropriate separation in the process of cell division (Siliakus, van der Oost *et al.* 2017).

The membrane fluidity is an important parameter in the maintenance of the physiological homeostasis of a bacterium, so when altered it must be adjusted again through the phenomenon denominated as “homeoviscous adaptation”. This phenomenon was firstly described as the cell membrane fluidity adjustment as response to a change in temperature, but rapidly was extended to other environmental stress situations (Sinensky 1974). The main strategies for adjusting membrane fluidity involve modifications in the FA composition of the cell membrane, such as changes in the degree of saturation, modifications of chain length, cyclisation, *cis* and *trans* isomerisation and *iso*- and *anteiso*-branching (Murinova and Dercova 2014; de Carvalho and Caramujo 2018).

In section 1.2, the mechanisms that occur at the membrane level under different conditions will be discussed.

### 1.1.3. Triacylglycerol biosynthesis by *Rhodococcus*

The occurrence of *Rhodococcus* species in all types of natural ecosystems is due to their metabolic strategies, including the production of different storage compounds. The production of storage compounds plays an important role in the survival during nutritional fluctuations that occurs under certain environmental conditions, once their accumulation can be used as a reserve of carbon and due to their extreme hydrophobicity, it is possible to be accumulated in large amounts in cells without changing the osmolarity of the cytoplasm (Hernández, M.A., Mohn *et al.* 2008). Storage compounds produced by rhodococci, and other bacteria, can be triacylglycerol (TAG), polyhydroxyalkanoates (PHA), wax esters, glycogen, trehalose-base lipids and polyphosphates (Alvarez, H.M., Silva *et al.* 2013). Among the storage compounds produced by *Rhodococcus,* the main compounds accumulated are TAG (Hernández, Martín A and Alvarez 2010).

TAG, also called triglycerides, are a type of lipid consisting of three long chain FA esterified to glycerol, which are non-polar and water-insoluble (Bruss 2008). The principal function of TAG is to act as storage compounds, but they could also act as regulator of cellular membrane fluidity by preventing unusual FA incorporation in the membrane phospholipids, or by acting as a sink for reducing equivalents (Alvarez, H.M. and Steinbüchel 2002). The accumulation of TAGs is more described in eukaryotic organisms, although this has been described in bacteria that belong to the genera *Rhodococcus, Mycobacterium, Streptomyces, Acinetobacter and Nocardia* (actinomycetes group). This class of lipids is the most important source of energy and FAs for membrane biosynthesis in cells under nutrient depletion conditions, because how they are less oxidized and have a high calorific value, compared to carbohydrates and proteins (Alvarez, H.M. and Steinbüchel 2002).

Bacteria can biosynthesize TAG through a wide range of carbon sources, including organic acids, n-alkanes, phenyl-alkanes, branched alkanes, mono- and disaccharides, aromatics and polyaromatic hydrocarbons, sugar, alcohols, oils, coal and agro- industrial wastes such as carob and orange wastes and sugar cane molasses (Alvarez, H.M. and Steinbüchel 2002; Alvarez, H.M., Silva *et al.* 2013). Depending on the source of carbon used, the variability of FA of TAG is high, but in general contain saturated and unsaturated straight long-chain FA, with a chain length between C14 and C18 (Alvarez, H.M. and Steinbüchel 2002; Alvarez, H.M. 2006).

The production of TAG is related to stressful conditions such as an excess of carbon source and simultaneously a limited availability of nitrogen source, indicating that the nutritional condition is the main influencer of TAG accumulation. This nutritional condition seems to be linked with the stationary phase or a response to some stress (Olukoshi and Packter 1994; Alvarez, H., Kalscheuer *et al.* 2000). Independently of the condition that induces the biosynthesis of TAG, their accumulation occurs as lipid droplets, which are surrounded by a membrane, in order to stabilize the hydrophobic content in the aqueous cytoplasm medium (Packter and Olukoshi 1995; Alvarez, Hector M, Mayer *et al.* 1996).

The strains able to accumulate a large quantity of TAG in cells after cultivation are considered as oleaginous. An example of an oleaginous strain is the *R. opacus* PD630*,* known as strain allowing the highest TAG accumulation, because is able to accumulate more than 85% of the cellular dry weight (CDW) as TAG (Alvarez, Hector M, Mayer *et al.* 1996). This oleaginous nature of rhodococci may play a significant role in biotechnological industry because these bacterial lipids may be used in the production of biotechnological products like additives for feed, cosmetics, lubricants, oleochemicals, biofuels and other manufactured products (Alvarez, H.M. and Steinbüchel 2010).

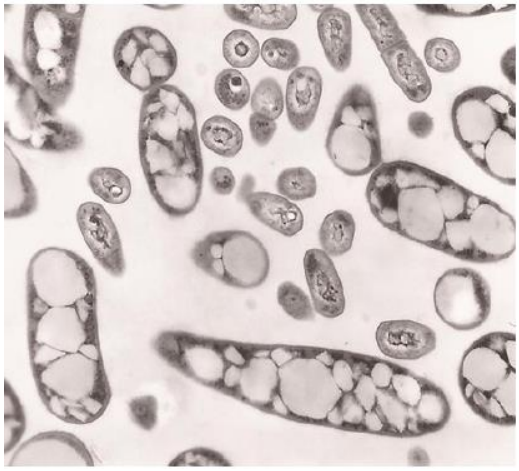

**Figure 3 –** Micrograph showing cells of *R. opacus* PD630 containing several TAG granules during growth on gluconate as the sole carbon and energy source (Figure taken from Alvarez, H.M., Silva *et al.* 2013).

### 1.1.4. *Rhodococcus erythropolis*

The *R. erythropolis* strain is one of the most studied strains within the genus *Rhodococcus*, due to its high tolerance to numerous conditions, and therefore its commercial interest has increased. Example of that is the use of the strain *R. erythropolis* UPV-1 in formaldehyde removal of industrial wastewater from a resin-manufacturing company (Hidalgo, Lopategi *et al.* 2002). The versatile metabolism of *R. erythropolis* combined with their proprieties of membrane, justify the potential of this bacterium in bioremediation processes and industry (de Carvalho and da Fonseca 2005b). Besides environmental applications and biosurfactants production, *R. erythropolis* has also been studied as antibiotic producer (Kitagawa, W. and Tamura, T. 2008; Teramoto, Kitagawa *et al.* 2009). An example of this is the production of a quinoline antibiotic by *R. erythropolis* JCM 6824 (Kitagawa, Wataru and Tamura, Tomohiro 2008).

The remarkable characteristics of *R. erythropolis* cells and their adaptation to different conditions of growth (different temperatures, pH values and presence sodium chloride and copper sulfate) have been widely demonstrated for the strain *R. erythropolis* DCL14 by de Carvalho (de Carvalho 2012). Besides the conditions referred previously, this bacterium is tolerant to water miscible solvents such as ethanol, butanol and dimethylformamide, but also to water-immiscible solvents such as dodecane, bis(2ethylhexyl) phthalate, toluene (de Carvalho, da Cruz *et al.* 2004; de Carvalho, Fatal *et al.* 2007), n-alkanes and alcohols (de Carvalho, Parreno-Marchante *et al.* 2005). All these characteristics show that this strain is highly adaptable and has enormous potential in bioremediation and biotransformation processes. (de Carvalho, Wick *et al.* 2009).

## 1.2. Adaptive mechanisms involved in *Rhodococcus* under several conditions

Environmental heterogeneity is an evolutionary challenge for organisms and therefore they need adaptation mechanisms to survive in hostile environments or in the presence of toxic compounds. In general, bacteria present the following mechanisms (Isken and de Bont 1998; de Carvalho, Poretti *et al.* 2005; de Carvalho, Wick *et al.* 2009):

I. Changes in lipid composition of cellular wall and membrane, and their permeability;
II. Modifications of the physicochemical properties of the cell surface (net surface change, hydrophobicity);
III. Cell aggregation and biofilm formation;
IV. Production of exopolymeric substances (EPS);
V. Degradation of toxic compounds and productions of secondary metabolites;
VI. Activation of efflux pumps.

Depending on environmental conditions, bacteria may use one or more of the mechanisms described above. With the objective to understand the mechanisms used by *Rhodococcus*, the following topics will discuss on the adaptations performed by this bacterium under different conditions.

### 1.2.1. Adaptation to changes in temperature

Temperature is one of the most important physicochemical parameters that affect the growth and development of the microorganisms. For this reason, the main challenge for microorganisms is to maintain the functionality of their macromolecules outside their optimal temperature range.

The membranes are generally in a liquid-crystalline structure, but depending on the temperature, it can be modified and, consequently the fluidity of the membranes is changed. The fluidity of a biological membrane depends on the lipid membrane phase-transition temperature (*Tm*), which indicates the temperature at which the membrane shifts from the preferred liquid-crystalline phase into a rigid gel phase (rigidification) or a fluid phase (fluidization) (Siliakus, van der Oost *et al.* 2017). The **Figure 4** shows the changes in the membrane structure and the behaviour of lipid bilayers depending on the temperature variation.

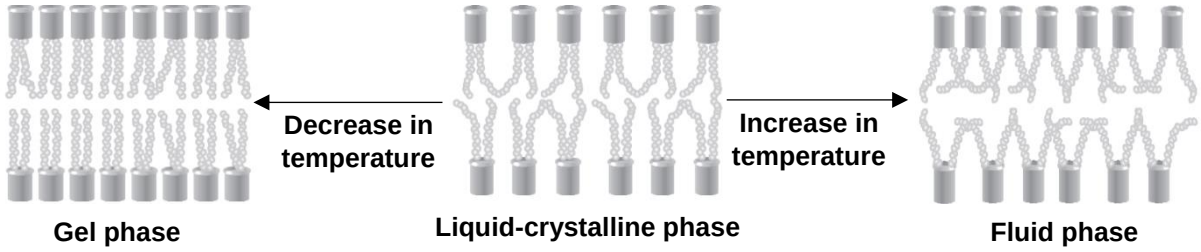


**Figure 4** – Schematic representation of changes in membrane structure due to changes in temperature (adapted from Los and Murata 2004).

As illustrated in **Figure 4**, when a decrease in temperature occurs, the FAs of the phospholipids of the cell membrane become more rigid, moving less, which causes a decrease in membrane fluidity and permeability. This decrease in membrane fluidity/permeability causes greater restriction on the entrance of hydrophobic molecules into the cells. On the other hand, when the temperature increases, the FA of phospholipids are changed, and the membrane becomes less rigid and therefore allows a greater movement of proteins and other molecules into and through the membrane, including harmful molecules (Los, Mironov *et al.* 2013; Siliakus, van der Oost *et al.* 2017). To counteract this, the cells are able to change the composition of the FA and the head-group of the phospholipids (Russell, N., Evans *et al.* 1995; de Carvalho and Caramujo 2018). If these modifications are sufficient the functionality and integrity of the membrane is maintained, otherwise the cells die.

In general, when exposed to lower temperatures, bacteria increase the amount of unsaturated FAs, decrease the chain length of fatty acyl chains, increase the amount of methyl branching and/or the ratio of *anteiso:iso*-branching, with the objective to increase the membrane fluidity to counterbalance the decreased caused by lower temperatures. Conversely, when exposed to higher temperatures, bacteria increase the amount of saturated, long-chain and branched chain *iso*-fatty acids with the objective to decrease the membrane fluidity to counterbalance the increased caused by higher temperatures. All these modifications are reviewed for example by (Beney and Gervais 2001; Siliakus, van der Oost *et al.* 2017; de Carvalho and Caramujo 2018).

A study performed by de Carvalho focused on the adaptability of the strain *R. erythropolis* DCL14 to temperatures regarded as extreme to the non-adapted cells (de Carvalho 2012). At the cellular membrane level, the adaptations of the cells resulted in an increase in degree of saturation of membrane FAs, when grown under non-optimal conditions in a temperature range from 4º C to 37º C. This increase was due to an increase in the relative percentage of SSFAs with a concomitantly decrease in the relative percentage of unsaturated FAs. In addition to that, it was observed a decrease in the relative percentage of saturated cyclopropyl and saturated methyl-branched fatty acids. At lower temperatures it was also shown, that the cells produced polyunsaturated fatty acids (PUFAs) (de Carvalho 2012).

In another study performed by Whyte *et al.* the strain *Rhodococcus* sp. Q15 was used to study alkane assimilation at low temperature (Whyte, Slagman *et al.* 1999). The authors referred that the analysis of the FA of the membrane showed a decrease in the degree of saturation, but also that this decrease was done in a lesser extent when the cells were grown at low temperature and in the presence of hydrocarbons. These results suggested that the strain modulates the membrane fluidity in response to the influence of low temperature and hydrocarbon toxicity (Whyte, Slagman *et al.* 1999).

A right level of membrane fluidity is also important, for the membrane proteins to continue to pump ions, take up nutrients and maintain cellular respiration. Protons and sodium ions have a key role in energy transduction, in both bacteria and archaea, and since previous studies suggested that the proton permeability of the membrane has a significative importance in cell viability, van de Vossenberg *et al.* proposed a theory. The theory proposed is defined as homeo-proton permeability, suggesting that bacteria and archaea adjust their membrane lipid composition, with the objective to maintain constant the level of proton permeability of the membrane independently of the growth temperature (van de Vossenberg, Driessen *et al.* 1999).

### 1.2.2. Adaptation to changes in pH

The physicochemical parameter pH is an important environmental factor, because it has a direct effect on microorganisms and exerts indirect effects in nutrient conditions and metal solubility and toxicity (Fernández-Calviño and Bååth 2010). The microorganisms are more sensitive to alterations in internal pH ($pH_i$) than in external pH ($pH_e$), although both may lead to a loss of viability. For growth and survival, most bacteria required a pH between 4 and 8, although some species may survive away from this range (Beales 2004). When a microorganism grows at pH values far from the optimal range, higher energy is required in comparison with the growth at optimal pH, because it is necessary to pump out or pump into the cell protons if the pH is low or high, respectively. This pump of protons is important for a correct functioning of cellular components (Booth and Kroll 1989; Brown and Booth 1991)

Both alkaliphiles and acidophiles, have as difficulty maintaining $pH_i$ as neutral (pH homeostasis), and it is important because most proteins have distinct range of pH within they can function (Krulwich, Sachs *et al.* 2011). The pH homeostasis of is obtained through the proton motive force (PMF). The PMF is an electrochemical gradient of protons ($H^+$) across the cell membrane, which is driven by pH gradient ($\Delta pH = pH_{in} - pH_{out}$). This pH gradient is an important energy supply, because during the PMF is produced chemical energy in the form of ATP (Siliakus, van der Oost *et al.* 2017). Acidophiles/Alkaliphiles have several pH homeostasis mechanisms, however the most effective strategy is the reduction of proton permeability by the plasma membrane (Krulwich, Sachs *et al.* 2011). In the presence of low pH there no exist a universal pattern of preferred FA composition, and in the **Table 2** some of the possible behaviours are presented.

**Table 2 –** Some of the possible behaviours presented by bacteria in low pH.

| Strain | Mechanism observed | Reference |
|---|---|---|
| ***Escherichia coli*** | Increased percentage of straight-chain fatty acids, decreased percentage of unsaturations and a lowered percentage of cyclopropane fatty acids | (Yuk and Marshall 2004) |
| ***Streptococcus mutans*** | Decreased percentage of short-chain fatty acids (C14:0 and C16:0, mainly) and an increased percentage of long-chain unsaturated fatty acids (C18:1 and C20:1, mainly) | (Fozo, Kajfasz *et al.* 2004; Fozo and Quivey 2004) |
| ***Lactobacillus casei*** | | |
| ***Listeria monocytogenes*** | Increased percentage of anteiso- and a decreased percentage of iso- branched chain fatty acids | (Giotis, McDowell *et al.* 2007) |

As observed in **Table 2**, there are different responses in FA composition of bacteria exposed to low pH, although the apparent appropriate response to maintaining the homeostasis seems to be the decreased membrane fluidity through an increase in C16:0 and cyclopropane C19:0 ω8c with a concomitant decrease in C18:1 ω7c (de Carvalho 2012). The increase of the cyclopropane is essential because decreases the proton permeability which equilibrate the pH (Lebre, De Maayer *et al.* 2017). Contrarily to acidophilic membranes, the studies in alkaliphilic membranes are scarce but a few general trends are observed like the enrichment in *iso*- and *anteiso*-branched chain fatty acids (BCFAs) and often a significant amount of MUFAs (Siliakus, van der Oost *et al.* 2017).

In the case of the genus *Rhodococcus,* a study performed by de Carvalho, using the strain *R. erythropolis* DCL14, focused on the adaptability of this strain further away from optimal values of pH (de Carvalho 2012). The response of cells to both low and high pH was an increase in degree of saturation of FAs of the cell membrane, while decreasing the relative proportion of BCFAs and saturated cyclopropyl-branched FAs (SCBFAs). This increase in degree of saturation resulted from an increase in the percentage of SSFAs (C18:0, mainly) and hydroxyl substituted FA with a simultaneously decrease in the relative percentage of unsaturated FAs. Besides these changes in FA composition, the cells responded to both low and high pH by increasing the zeta potential (de Carvalho 2012).

### 1.2.3. Adaptation to the presence of salt

In marine environments there are usually variations in salinity, although they cause some stress in the microorganism, because the integrity and hydration of the cell are related to the solute content and osmotic pressures of their environment (Wood 2015). Bacteria are constantly in contact with the surrounding environment and, due to the membrane permeability to water, are in balance with the environment. If there are alterations in external osmotic pressure, water fluxes will occur with the objective to re-establish the balance between the intracellular concentration of bacteria and their surroundings. However, this water fluxes disturb many properties of the cells, such as volume, turgor pressure, cell wall, and cytoplasmic membrane and salt ion concentration. Bacteria respond to these variations in external osmotic pressure, by accumulating or releasing solutes, designated as compatible solutes (Gutierrez, Abee *et al.* 1995).

Compatible solutes or osmoprotectants are nontoxic inorganic salt or organic molecules that are accumulated in the cytoplasm and have as function to increase/decrease the internal osmolality ensuring water uptake/release (Gutierrez, Abee *et al.* 1995). These molecules, either imported or synthesized, could be sugars (*e.g.* sucrose and trehalose), cations (*e.g.* $K^+$), polyols, amino acids and their derivatives (*e.g.* proline, glutamate, ectoine and glycine betaine), and quaternary compounds (Csonka and Hanson 1991; Galinski 1995).

In addition to the accumulation of compatible solutes, in the presence of high salinity concentrations, there are modifications in the composition of the cell membrane to maintain fluidity, and contrary to what happens with changes in temperature, the main response of the bacteria occurs in the head-group composition of the phospholipids, instead in their FA composition (Beales 2004). The common behaviour is an increase in the content of anionic phospholipids compared with the neutral

phospholipids, more specifically due to an increase of the percentage of cardiolipin (CL) instead of phosphatidylglycerol (PG) (Russell, Nicholas J 1989).

Regarding the genus *Rhodococcus*, studies performed by de Carvalho and co-workers, using the strain *R. erythropolis* DCL14 in the presence of salt, showed that this strain, increase the amount of SSFAs and hydroxy-substituted while decrease the other FAs (de Carvalho 2012; de Carvalho, Marques *et al.* 2014). This alteration in FA composition resulted in a decrease in membrane fluidity and permeability as in halotolerant strains. Beyond this, the strain is able to synthesize PUFAs through a fast post *de novo* synthetic modification of MUFAs, due to expression of desaturases, because this production of PUFAs was accompanied by a concomitantly decrease of MUFAs (de Carvalho 2012; de Carvalho, Marques *et al.* 2014). In relation to the strain *R. opacus* PWD4, when in the presence of salt, there was an increase in the ratio of MA over phospholipid FAs (PLFA), a decrease in the average chain length of MA and an increase in the degree of saturation of PLFA. These modifications led to a decrease in membrane fluidity, a more hydrophobic MA, and an increase in cell surface hydrophobicity (de Carvalho, Fischer *et al.* 2016).

### 1.2.4. Adaptation to the presence of metals

Heavy metals can be found in the environment due to volcanic activity or other natural geological events, but also through anthropogenic releases such as antimicrobials/pesticides releases, fossil fuel burning, mining, smelting or other industrial application (Hobman and Crossman 2014). These metals can be divided in two classes: essential metals such as calcium, cobalt, copper, potassium, sodium, zinc or nonessential metals such as aluminium, cadmium, gold, mercury, silver. The first class of metals plays an important role in the microorganism's life, since they provide vital cofactors for metalloproteins, enzymes or biochemical reactions; stabilizes protein structure and bacterial cell wall; regulate gene expression and help in maintaining osmotic balance. The second class of metals has no biological role and can be toxic for bacteria (Bruins, Kapil *et al.* 2000).

Besides nonessential metals, high concentrations of essential metals are also toxic to microorganisms, because enzyme inactivation and cell damage may occur. This toxicity occurs due to the displacement of essential metals from their native binding sites. In relation to nonessential metals, they bind with higher affinity than essential metals to thiol-containing groups and oxygen sites (Hughes and Poole 1989; Poole and Gadd 1989). The natural exposure of bacteria to both types of metals, influence bacteria to develop mechanisms to acquire essential metals, but also to control their intracellular levels and to eliminate those that in excess and/or are harmful to the cell (Hobman and Crossman 2014). These resistance mechanisms are related to blocking the metal into the cell such as changes in permeability barrier or extracellular sequestration, as well as detoxification of the metals inside the cell through the active transport of the metal from the cell, enzymatic conversation, alteration of target sites or intracellular sequestration (Bruins, Kapil *et al.* 2000).

Bacteria that naturally is capable to form an EPS coating can bioabsorb metal ions, preventing the uptake of metals and their interactions with vital cellular components (Scott and Palmer 1990; Scott, Sage *et al.* 1998). Intracellular sequestration, is a process of confining metals inside the cytoplasm, preventing their exposure to essential cellular compounds, while extracellular sequestration is the

accumulation of metals by cellular components in the periplasm or the outer membrane (Bruins, Kapil *et al.* 2000). In relation to the active transport or efflux systems, it is the highly specific export of toxic metal from the cytoplasm of microorganisms and can be non-ATPase or ATPase-linked. Lastly the enzymatic detoxification consists in the conversion of the metal into a chemical species less toxic or more volatile through oxidation, reduction, methylation or demethylation (Williams and Silver 1984; Bruins, Kapil *et al.* 2000).

Regarding the genus *Rhodococcus*, a study performed by de Carvalho, using the strain *R. erythropolis* DCL14 focused on the adaptability of this strain to the presence of copper sulfate (de Carvalho 2012). The cells responded with a different behavior depending on the concentration of metal, but in general there was an increase in the degree of saturation of membrane FAs and a decrease in relative percentage of SCBFAs and BCFAs with increasing copper concentrations. The two latter classes of lipids were not produced for copper sulfate concentrations of 0.25% and higher. In contrast, it was observed an increase in the relative percentage of hydroxyl substituted and PUFAs with increasing cooper concentrations. It was also observed an increase in the zeta potential of the cells with increasing cooper sulfate concentrations (de Carvalho 2012).

### 1.2.5. Adaptation to the presence of solvent organics

Organic solvents like antimicrobial agents (*e.g.* alcohols, aromatics, phenols) are known to be extremely toxic to cells, even at very low concentration as 0.1% (v/v) (Hugo 1978; Lucchini, Corre *et al.* 1990), because they accumulate in membrane and increase its fluidity (Sikkema, de Bont *et al.* 1995). This incorporation and accumulation into cell membrane disturb the interactions lipid-lipid and lipid-water existing, affecting its structural and functional integrity leading to the disruption (Weber and de Bont 1996).

The toxicity of a solvent is correlated with its partition coefficient in an equimolar system of *n*-octanol:water, which is defined as log $P_{ow}$ value (Laane, Boeren *et al.* 1987). According to this scale, enzymes and microorganisms present a minimum of activity with solvents with log $P_{OW}$ values between 0 and 2 and between 2 and 4, respectively, while solvents with a log $P_{OW}$ value higher than 4, it will result in increased biocatalyst stability (Laane, Boeren *et al.* 1987). However, the actual membrane concentration of the solvent in the bacterial cell membrane depend on the solvent concentration in the water phase but also on the partitioning of the solvent from the water phase to the membrane. In 1994, Sikkema and co-workers proposed a correlation found between the log $P_{OW}$ value of a solvent and its partitioning between the membrane and water (log $P_{MW}$) (**Equation 1**) (Sikkema, Weber *et al.* 1994).

$$\log P_{\mathrm{M/W}} = 0.97 \times \log P_{\mathrm{O/W}} - 0.64 \qquad (1)$$

Normally, solvents with a log $P_{ow}$ value between 1 and 4, have a high solubility in water, but are also able of partitioning into biological membranes. This partitioning into membranes causes a high accumulation of these solvents in the cytoplasmic membrane of the bacterium, which is extremely toxic and leads to its rupture. In relation to solvents with a log $P_{ow}$ value above 4 (hydrophobic solvents), they are accumulated in the membrane, but since the water solubility is lower, they are not toxic to the cells. Concluding, the lower log $P_{ow}$ value, the greater its solubility in water and consequently the more toxic the solvent will be (de Bont 1998).

Solvent toxicity is not only related to the inherent toxicity of the compound but also with the capability of the bacteria to tolerate these toxic compounds. Bacteria that can assimilate these toxic compounds are considered as organic solvent–tolerant bacteria. The first report of an organic solvent-tolerant bacteria was in 1989 by Inoue and Horikoshi, who discovered the strain *Pseudomonas putida* IH-200, capable of growing in the presence of toluene (log $P_{ow}$ =2.5 (Sardessai and Bhosle 2002)), although it did not metabolize tolueno as a source of carbon (Inoue and Horikoshi 1989). Since this discovery, new strains of gram-positive bacteria have shown high tolerance for organic solvents more toxic than toluene, like strains belonging to the genus *Rhodococcus* (de Carvalho 2010).

In order to survive in the presence of organic compounds, bacteria need to develop mechanisms to overcome solvent stress, and several reviews have been published where are discussed the mechanisms of cell adaptation for both gram-negative and gram-positive bacteria (Sikkema, Weber *et al.* 1994; de Bont 1998; Isken and de Bont 1998; Segura, Duque *et al.* 1999; Ramos, Duque *et al.* 2002; Sardessai and Bhosle 2002; Segura, Duque *et al.* 2002; Fernandes, Marques *et al.* 2011; Torres, Pandey *et al.* 2011; Segura, Molina *et al.* 2012; Joo 2015). When exposed to organic solvents the bacteria can responded through the following mechanisms: (1) changes in the cell membrane to modulate its fluidity, (2) metabolism or inactivation of the toxic compound, (3) increased efflux of the toxic compound, (4) biofilm formation.

In relation to the gram-positive bacteria, different physiological responses could be observed in different strains. In the specific case of *Rhodococcus* strains, the general response to the presence of organic solvents are the following: changes in the degree of saturation of the membrane fatty acids, changes in the length of the FAs, or changes in the MA content (de Carvalho 2010). In **Table 3**, the mechanisms observed at the cellular membrane level in some strains of *Rhodococcus* are shown.

**Table 3** – Mechanisms of solvent tolerance observed in some *Rhodococcus* strains (adapted from de Carvalho 2010).

| Strain | Compound | Mechanism observed | Reference |
|---|---|---|---|
| ***Rhodococcus* sp. 33** | Benzene | Increased percentage of C16:0 and 10MeC18:0 while decreased C16:1 | (Gutiérrez, Nichols *et al.* 1999) |
| ***R. opacus* GM-14, GM-29, 1CP** | Benzene, phenol, 4-chlorophenol, chlorobenzene or toluene | Increased percentage of branched (10-methyl) fatty acids | (Tsitko, Zaitsev *et al.* 1999) |
| ***R. opacus* PWD4** | 4-chlorophenol | Decrease in average chain length of MA and increase in degree of saturation | (de Carvalho, Fischer *et al.* 2016) |
| ***Rhodococcus sp. 20, R. opacus* PD360, *R. erythropolis 17*** | Pentane, hexadecane | Incorporation of fatty acids corresponding to the chain length of the substrate used | (Alvarez, H.M. 2003) |
| ***R. erythropolis* CCM 2595 and *R. erythropolis CCM 2595 pSRK 21* (genetically modified)** | Phenol and humic acid | Increase in degree of saturation | (Kolouchová, Schreiberová *et al.* 2012) |

**Table 3** – Mechanisms of solvent tolerance observed in some *Rhodococcus* strains (adapted from de Carvalho 2010) (continuation).

| Strain | Compound | Mechanism observed | Reference |
|---|---|---|---|
| ***R. erythropolis* DCL14** | Short-chain alcohols *n*-alkanes and long-chain alcohols | Decrease in degree of saturation and increase in degree of saturation | (de Carvalho, Parreno-Marchante *et al.* 2005) |
| | Toluene | Increase percentage of C14:0 and C16:0 while decreased C18:0 and increased percentage of iso-branched while decreased straight-chain fatty acids | (de Carvalho, Fatal *et al.* 2007) |
| | C5-C16 *n*-alkanes | Incorporation of saturated fatty acids corresponding to the chain length of the substrate used;<br>Net surface charge increased with chain length | (de Carvalho, Wick *et al.* 2009) |

In **Table 3**, it is possible to observe that the response of *Rhodococcus* to the presence of alcohols is different depending on the type of the alcohol. If the cells are grown in presence of short-chain alcohols, a decrease in the degree of saturation is observed, due to the insertion of unsaturated FAs (de Carvalho, Parreno-Marchante *et al.* 2005). This insertion results from a slightly penetration of this alcohols into the hydrophobic phospholipid bilayer that cause an enlargement effect on the hydrophilic headgroups. In contrast, if the cells are grown in presence of long-chain alcohols, an increase of the degree of saturation is observed, due to the penetration of the alcohol into the cell membrane (de Carvalho, Parreno-Marchante *et al.* 2005).

Moreover, it was shown that there is a relationship between the chain length of the carbon source and the FA profile. Usually, cells grown on *n*-alkanes incorporated SSFAs corresponding to the chain length of the substrate used into their membrane (Alvarez, H.M. 2003). Alvarez showed that most FAs presented in actinomycetes bacteria were correlated to the chain length. This suggests that the substrates might be incorporated into cellular lipids after monoterminal oxidation and without complete degradation to acetyl-CoA. The author further suggests that hydrocarbons should be used under nitrogen-limited conditions, in the biosynthesis of triacyclglycerols (Alvarez, H.M. 2003).

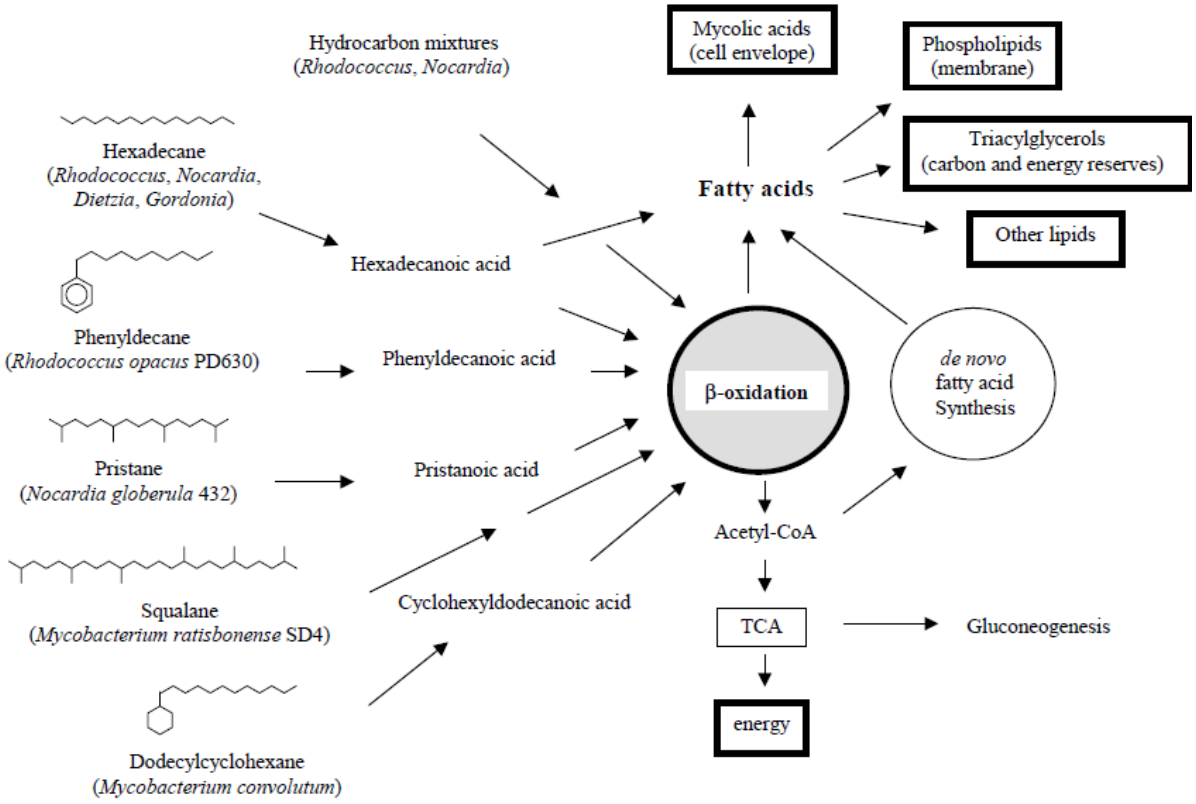


**Figure 5** – Degradation and assimilation of different hydrocarbons by non-sporulating actinomycetes bacteria. The metabolism of no-sporulating actinomycetes adapts in response to the availability of hydrocarbons with an alkyl side chain in their structure for the production of a large variety of lipids with different complexity and functions. Abbreviations: TCA: tricarboxylic acid cycle (This figure was published in International Biodeterioration & Biodegradation, Vol 52, Héctor M. Alvarez, Relationship between β-oxidation pathway and the hydrocarbon-degrading profile in actinomycetes bacteria, Pages 35-42, Copyright © 2002 Elsevier Science Ltd. (2003)).

Another mechanism that allows bacterial strains to thrive in the presence of organic solvents is the bioconversion or metabolization of these compounds. Several studies made by de Carvalho *et al.* showed that the cells of *R. erythropolis*, when in two-phase systems, due to their hydrophobicity, migrated towards the organic phase, and were found partitioned between aqueous and organic phases, but also inside the solvent droplets (de Carvalho and da Fonseca 2002a;b;2003). This allows the direct access of the cells to the solvent layer containing organic compounds. When cells are directly inside the organic phase, there is easier access the dissolved hydrophobic substrates in both biocatalytic and bioremediation processes. Several other reports are described in a review made by de Carvalho (de Carvalho 2010).

The EPS produced by some strains is another mechanism that could provide tolerance to organic solvents, since they lower cell surface hydrophobicity. In fact, Kobayashi and co-workers showed that low cell surface hydrophobicity could be a defense mechanism against these toxic compounds (Kobayashi, Takami *et al.* 1999). Cells of *R. rhodochrous* present a non-EPS and an EPS producer variant. When exposed to 10% (v/v) of *n*-hexadecane, the mucoidal strain was resistant to the presence of *n*-hexadecane, while the reverse was observed on the rough strain (Iwabuchi, Sunairi *et al.* 2000). However, when the EPS produced by mucoidal strain was added to cultures of the rough strains, rough

cells acquired resistance to the presence of *n*-hexadecane, indicating that EPS confer tolerance to the genus *Rhodococcus*, in the presence of *n*-hexadecane (Iwabuchi, Sunairi *et al.* 2000). Other studies showed equivalent results, such as the resistance acquire for mucoidal strain of *Rhodococcus* sp. 33 and *R. rhodochrous* S-2 to organic compounds, through the production of an EPS (Iwabuchi, Sunairi *et al.* 2002; Aizawa, Neilan *et al.* 2005) or the resistance acquire for *R. erythropolis* PR4 to pristine, a C19 branched alkane (Urai, Yoshizaki *et al.* 2007). In the later study the resistance acquires allowed the strain to degrade the alkane (Urai, Yoshizaki *et al.* 2007).

Some of the EPS produced by several rhodococci strains are biosurfactants, usually glycolipids, such as in *R. equi* Ou2 or *R. erythropolis* 51 T7 (de Carvalho 2010). In the presence of C5-C16 *n*-alkanes, *R. erythropolis* DCL14 produced, when grown in *n*-alkanes with longer carbon chain, a biosurfactant that significantly decreases surface tension of the culture medium (de Carvalho, Wick *et al.* 2009). In the same study, it was also observed that the cell that did not produce biosurfactant, formed clusters in the presence of *n*-alkanes with longer carbon chain, which is a common pattern for non-EPS producer bacteria when grown in these compounds (de Carvalho, Wick *et al.* 2009).

The formation of biofilms or the activation of efflux pumps are also defense mechanisms. Efflux pumps are membrane transport proteins that mediate the evasion of nontoxic compounds from the interior of the cells and are responsible to the efflux of organic solvents from inside the cell (de Carvalho, Costa *et al.* 2014). Biofilms protect the cells against toxic compounds, because when the cells are inside the biofilm matrix there an impediment of mass transport of toxic compounds to the cells, being the cells less susceptible to the compounds (Heipieper, Keweloh *et al.* 1991). An example of this was observed by de Carvalho and co-workers for the strain *R. erythropolis* DCL14 grown in biofilm and in cell suspension (de Carvalho, Wick *et al.* 2009). The authors observed much lower toxic effect of *n*-octane on biofilm cells, while planktonic cells were strongly inhibited by the accumulation of 1-octanol, a metabolite of the alkane monooxygenase during growth on *n*-octane (de Carvalho, Wick *et al.* 2009).

### 1.2.6. Adaptation to desiccation

For the soil microorganism, drought is perhaps the most common environmental stress since one third of the earth is cover by arid, semi-arid, or seasonally arid zones, as well as, other ecosystems that have regular drought or episodic dry/rewetting cycles, like Mediterranean ecosystems (Schimel, Balser *et al.* 2007; Barnard, Osborne *et al.* 2013). This condition can be caused either by a lack of water (desiccation) or excessive solute concentration (hypertonicity) and to reverse the negatives morphological, physiological and biochemical consequences of water depletion, some organisms have developed a set of adaptive strategies to survive. In general, research has been mainly focused on gram-negative (Lebre, De Maayer *et al.* 2017), but most of the mechanisms used by bacteria in drought conditions are similar to those used in the presence of high amounts of salt, since in both conditions there are loss of water.

The cellular membrane of bacteria is harshly affected by changes in external water availability, because it is the main barrier between the intracellular and extracellular environment, and for that changes in composition of membrane phospholipids and FAs is crucial for cell survival (Lebre, De Maayer *et al.* 2017). Besides, changes in cell membrane to modulate its fluidity and preserve during

extreme desiccation the membrane in a liquid crystalline phase, other mechanisms ensure the cell survival such as: EPS production, biofilm formation and accumulation of compatible solutes (Lebre, De Maayer *et al.* 2017). In relation to the biofilms, they confer protection against dynamic environments due to the hygroscopic nature of the majority compound (EPS) that confers a highly hydrated environment, the cells being protected against fluctuation in water content (Flemming, Wingender *et al.* 2016; Esbelin, Santos *et al.* 2018).

The accumulation of compatible solutes is also important in extreme desiccation because it restores the osmotic equilibrium and permits the functioning of proteins, even with low water activity (Potts 1994). That hydration is achieved through the mechanism "Preferential Hydration and Exclusion", where the solutes compete with the water to bond to the biomolecules and membranes, through hydrogen bonds (Arakawa, Carpenter *et al.* 1990; Potts 1994). When occurs the rehydration, according with the "Water Replacement Hypothesis", the disaccharide trehalose and sucrose are important stabilizers because prevent the membrane disruption and protein denaturation, enabling even in a low water content, the membrane in a liquid-crystalline state (**Figure 6**) (Crowe, Crowe *et al.* 1997; Crowe, Carpenter *et al.* 1998).

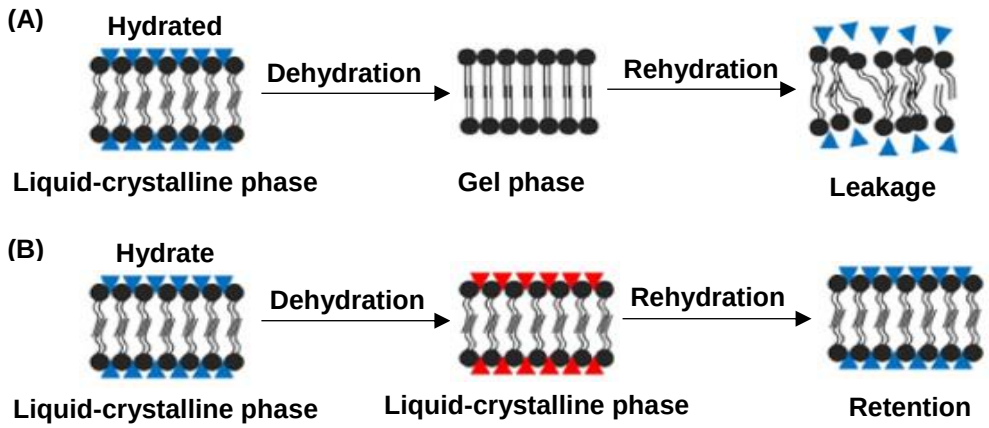


**Figure 6 –** Water replacement hypothesis. Legend: **(A) –** Dehydration without stabilizers; **(B) –** Dehydration with stabilizers; **Blue triangles –** water; **Red triangles –** stabilizers (*e.g.* trehalose, sucrose) (Figure adapted from Rozsypal 2015).

Regarding the genus *Rhodococcus*, there are two studies performed. The first study was made by Alvarez *et al.* using the strain *R. opacus* PD630 in water stress (Alvarez, H.M., Silva *et al.* 2004). For this strain under dehydration conditions the following mechanisms were observed: (1) synthesis and accumulation of compatible solutes, (2) alterations in MA, and (3) production of EPS. The compatible solutes synthesised were: trehalose, ectoine and hydroxyectoine. However, the authors expected that it was glycerol the compatible solute synthesised, because under dehydration conditions the strain utilized TAG as endogenous carbon and energy sources, and since glycerol can be generated through TAG degradation, it would be an accessible compatible solute in the cells of *R. opacus* PD630. This TAG biosynthesis was not only for energy source but also as precursors for biosynthesis of different MA which contribute to cell envelope adaptation. This strain also produced an EPS matrix as reservoir of water, reducing the water loss of the cells (Alvarez, H.M., Silva *et al.* 2004).

The second study was made by LeBlanc *et al.,* using the strain *R. jostii* RHA1, and as in the first study, the cells synthetized and accumulated trehalose and ectoine as compatible solutes. These two studies may suggest that these solutes may be prevalent in rhodococci under water stress conditions (LeBlanc, Gonçalves *et al.* 2008).

In fact, Warton *et al.* identified 11 isolates of *Rhodococcus* spp. that could resist to dry heat treatments and recover their degrading ability following dehydration (Warton, Matthiessen *et al.* 2001), suggesting an adaptation to these environments. Therefore, understand the mechanisms that contribute to that resistance will enrich our knowledge not only of environmental applications but also at public health level, since there are some reports of pathogenic bacteria that have been found to survive and thrive in preserved food, skin and clothing (Lebre, De Maayer *et al.* 2017).

## 1.3. Objectives

Bacteria such as *Rhodococcus* are found under various stress conditions, such as drought stress, since they inhabit several conditions. Previous studies carried out in the laboratory of de Carvalho have shown that *R. erythropolis* cells may adapt to *e.g.* organic solvents, low and high temperature and pH values, and high concentration of salt and metal ions. However, the behaviour of this strain under desiccation and the role of lipids during cell adaptation have not been studied. The objectives of the master studies were thus the following:

1. Understanding the role of the lipids in the tolerance of *R. erythropolis* cells to dehydration;
2. Study of the effect of growth conditions such as medium composition, age of the cells at the time of exposure to dryness, and the mode of growth (planktonic vs biofilm) on tolerance;
3. To assess other phenotypic modifications occurring during adaptation;
4. To evaluate the role of storage lipids in cell tolerance to dehydration and antibiotic exposure.

# 2. Material and Methods

## 2.1. Bacterial Strain

*Rhodococcus erythropolis* DCL14 was first isolated from a sample from a ditch in Reeuwijk (van der Werf, Swarts *et al.* 1999). It is stored and maintained at the Institute for Bioengineering and Biosciences (iBB), Instituto Superior Técnico. The non slime-producing variant, isolated from the slime-producing variant at IST, was used in this study.

## 2.2. Growth of planktonic cells

*R. erythropolis* DCL14 colonies selected from agar plates were used to inoculate 100 mL Erlenmeyer flasks closed with rubber bungs, containing 20 mL of mineral medium (MM) supplemented with 0.25% (v/v) of absolute ethanol (Panreac) as carbon source (pre-inoculum). The MM is composed of the following compounds per litre of demineralised water: 0.01 g EDTA, 0.002 g $ZnSO_4 \cdot 7H_2O$, 0.001 g $CaCl_2 \cdot 2H_2O$, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.0002 g $Na_2MoO_4 \cdot 2H_2O$, 0.0002 g $CuSO_4 \cdot 5H_2O$, 0.0004 g $CoCl_2 \cdot 6H_2O$, 0.001 g $MnCl_2 \cdot 4H_2O$, 0.1 g $MgCl_2 \cdot 6H_2O$, 2 g $(NH_4)_2SO_4$, and 1.55 g $K_2HPO_4$ and 0.85 g $NaH_2PO_4 \cdot H_2O$ for buffering (all chemical were from Sigma Aldrich) (Cortes and de Carvalho 2015).

Depending on the assay, the cells were cultured in following media: Mueller-Hinton Broth (MH, Fluka Analytical); and MM with and without salt (35 g/L NaCl, Carl Roth) supplemented with 0.25% (v/v) absolute ethanol as carbon source. The cultures were incubated at 30º C and 200 rpm on an incubator Agitorb 200 (Aralab). Cell growth was monitored by optical density measurements at 600 nm ($OD_{600}$) using a T70 UV/VIS Spectrophotometer (PG Instruments Ltd.) and all growths were performed in duplicate.

## 2.3. Growth of biofilm cells

*R. erythropolis* DCL14 cells were grown on 24-well plates containing 1 mL of cell suspension with an initial $OD_{600}$ of *ca.* 0.5-0.8. The cells, in these assays, were cultured in MM with and without salt, supplemented with 0.25% (v/v) of absolute ethanol as carbon source, and the plates were incubated at 30º C and 100 rpm.

## 2.4. Dehydration assay

Once planktonic cells reached mid-exponential or stationary phase, 1 mL of cell suspension were collected to 1.5 mL eppendorf tube (Eppendorf, Hamburg, Germany) and centrifuged (µSpeedFuge SFA13K Microcentrifuge, Savant Technologies) at 10,000 rpm for 5 min. The supernatant was discharged, the pellet was resuspended in 1 mL of milli-Q water, to wash the cells, and the resulting suspension was centrifuged at 10,000 rpm for 1 min. The supernatant was removed, and the pellet was placed on a vacuum dry evaporation system (RapidVap from Labconco, Kansas City, MO, USA).

Regarding biofilms cells, after *ca*. 24 h of cultivation, the cells from each well were resuspended in 1 mL of MM with and without salt and the resulting cell suspension was transferred to a 1.5 mL eppendorf tube (Eppendorf, Hamburg, Germany), and the procedure described above for planktonic cells was followed.

The RapidVap evaporator was programmed at a temperature of 30º C, without agitation, and a vacuum of 70 mbar. To assess the effect of desiccation in *R. erythropolis* cells, they were collected after 0, 5, 10, 20, 40, 60, 120 and 180 min of drying. At each sampling time, samples for analysis of lipids and dry weight determination were collected. Cell dry weight was determined after 24 h at 65º C using a Mettler Toledo AG104 balance. To determine the effect of rehydration, the pellets of planktonic cells in a 1.5 mL Eppendorf tube were rehydrated with 1 mL of the corresponded growth medium during 1h after drying. Regarding rehydrated biofilm cells, the procedure of dehydration, was different from those that were not rehydrated. After *ca*. 24 h of cultivation, the growth medium was removed with a disposable syringe with careful to not lose biofilm cells. The plate was covered with a Breathe-Easy® sealing membrane (Sigma-Aldrich) and was placed on RapidVap evaporator. After a given time, part of the membrane was cut and removed with the objective of having the drying times mentioned above. After the dryness period, 1 mL of the corresponded growth medium was added to each well and the biofilm cells were rehydrated during 1 h. All assays were performed at least in duplicate.

## 2.5. Fatty Acid Composition

The FA composition of dehydrated planktonic and biofilm cells was determined immediately after the desiccation period. Regarding rehydrated planktonic cells, after 1h of rehydration each 1.5 mL eppendorf tube (Eppendorf, Hamburg, Germany) with 1 mL of cell suspension was centrifuged (µSpeedFuge SFA13K Microcentrifuge, Savant Technologies) at 10,000 rpm for 5 min. The supernatant was discharged, the pellet was resuspended in 1 mL of milli-Q water, to wash the cells, and the resulting suspension was centrifuged at 10,000 rpm for 1 min. The supernatant was removed, and the FA composition was determined. Concerning rehydrated biofilm cells, after 1 h of rehydration the cells were resuspended, 1 mL of cell suspension were collected to 1.5 mL eppendorf tube (Eppendorf, Hamburg, Germany), and was followed the procedure described above for rehydrated planktonic cells.

The extraction and methylation of the FAs from the cells to fatty acid methyl esters (FAMEs) was simultaneously done using the Instant FAMES kit from MIDI, Inc (Newark, DE, USA). The analysis of FAMEs was made on a 6890N gas chromatograph (GC) from Agilent Technologies (Palo Alto, CA, USA), which has a flame ionization detector (FID) and a 7683 B series injector, and is equipped with a 25 m long Agilent J&W Ultra 2 capillary column from Agilent. The software used to program and control the equipment was the Sherlock software package, version 6.2, from MIDI, Inc. Analysis of the lipid profile was made using the PLFAD1 method and the identity of the FAMEs was achieved by comparison of the retention times with MIDI calibration standards.

The FAs were grouped into classes, to better understand the differences in the lipid composition of the cells, according to their chemical structure: saturated straight chain (SSFAs), monounsaturated (MUFAs), polyunsaturated (PUFAs), saturated *iso*- and *anteiso*-methyl branched (BCFAs), 10-methyl branched (10MBFAs), saturated cyclopropyl-branched (SCBFAs), dimethylacetals (DMAFAs) and other FAs (FA that do not belong to the previously mentioned classes). The degree of saturation (Dsat) of the FAs of the phospholipids of the cell membrane was defined as the ratio between the total SSFAs and total MUFAs.

## 2.6. Zeta Potential

To determine the net surface charge of the cells, 20 µL of cell suspension were collected after 0, 60, 120 and 180 min of desiccation from the samples that were rehydrated immediately after the addition of media, and after 1h of rehydration. Each sample was suspended in 2 mL of $KNO_3$ (10 mM) and the electrophoretic mobility of the *R. erythropolis* DCL14 cell suspension was determined in a Doppler electrophoretic light scattering analyser (Zetasizer Nano ZS, Malvern Instruments Ltd.) using a clear disposable zeta cell. The potential zeta ($\zeta$) was calculated using the electrophoretic mobility as and indirect measure of the cell surface charge, according to the method of Helmholtz-von Smoluchowski (Hiemenz and Rajagopalan 1997). Calculations were automatically made using the Zetasizer software version 7.12, form Malvern Instruments.

## 2.7. Cell Viability

Cell viability of dry samples was assessed using a Live/Dead® *Bac*Light™ bacterial viability kit from Molecular Probes (Invitrogen Co., Carlsbad, California, USA) as described previously (de Carvalho and da Fonseca 2003). After suspension of cells from dried pellets in 1 mL of MM, 250 µL of cell suspension were collected to an eppendorf tube and the cells were stained using the viability kit referred previously. This viability kit contains a mixture of SYTO®9 green fluorescent nucleic acid stain and propidium iodide. Viable cells were stained green by SYTO®9 and non-viable cells were stained red since propidium iodide only enters in cells with damaged membranes. The cells of *R. erythropolis* DCL14 were observed by fluorescence microscopy using an Olympus CX40 microscope, with an Olympus U-RFL-T burner and an U-MWB mirror cube unit (excitation filter: B450-480; barrier filter: BA515). The capture of images was made by an Evolution™ MP5.1 CCD colour camera using software Image-Pro Plus, both from Media Cybernetics, Inc. (USA). At least 15 imagens were taken from each sample. Image analysis was performed as described previously (de Carvalho, Pons *et al.* 2009).

## 2.8. Accumulation of storage lipids

Accumulation of storage lipids in droplets and the production of exopolymeric glycolipids were promoted in *R. erythropolis* by growing the cells with glucose and trehalose as carbon sources at concentrations of 2.5, 5.0 and 10.0 g/L. After 24 h of cultivation, three samples of 1 mL of each culture was collected with the objective of determining: i. the tolerance of the cells containing the lipid droplets to dehydration; ii. the tolerance of these cells to antibiotics, more specifically to vancomycin, present at different concentrations, and iii. the alteration in lipid composition of the cells. The dehydration assay and the FAs analysis were performed as previously described in sections 2.4 and 2.5, respectively. All assays were performed in duplicate.

### 2.8.1. Tolerance of the cells to antibiotics

#### 2.8.1.1. Determination of the Minimal Inhibitory Concentration (MIC)

The MICs were determined using an Oxoid M.I.C.Evaluator™ (M.I.C.E™; Thermo Fisher Scientific) which comprises an antibiotic gradient from 0.015-256 µg/mL on a support. The assay was performed in Tryptic Soy Agar (TSA, Fluka Analytical) plates, and 100 µL of a cell culture diluted to 0.5 McFarland standard was spread and a M.I.C.E. strip was added. The plates were incubated at 30º C during 16-18 h and the MIC was determined by observation of the lowest concentration that inhibited cell growth.

#### 2.8.1.2. Time-Dependent Killing

To 15 mL falcon tubes (Thermo Fisher Scientific), 1 mL of *R. erythropolis* DCL14 exponentially growing cells and vancomycin at a concentration of 2 µg/mL were added. The tubes were incubated at 30º C and 200 rpm and at certain time intervals (before the addition of vancomycin; 2.5 h and 5 h after addition) a tube was taken from the incubator, and the cells were collected by centrifugation at 3,500 rpm for 5 min. The supernatant was discharged, and the pellet resuspended in 1 mL of milli-Q water to wash the cells, and centrifuged again at 3,500 rpm for 5 min. The supernatant was removed, and the pellet was resuspended in 1 mL of fresh MM containing no vancomycin. This cell suspension was then sequentially diluted in $10^{-1}$ steps in MM up to $10^{-6}$, using sterile eppendorfs and aseptic techniques. The dilutions $10^{-4}$, $10^{-5}$ and $10^{-6}$ were vortexed and 20 µL of each sample were aseptically transferred and spread on Tryptic Soy Agar (TSA, Fluka Analytical) plates. The plates were then incubated at 30º C and the colony-forming units (CFUs) were counted after *ca.* 16-18 h. To confirm if the cells were dead or requiring longer time for growth, the plates were kept at 30º C for up to 48 h.

# 3. Results and Discussion

With the aim of finding the role of lipids in *R. erythropolis* DCL14 during adaptation to dehydration the FA profile was analysed. To assess if growth conditions could influence the behaviour of the cells, they were grown in three different media (MH and MM with and without NaCl). The cells were collected at different of cultivation, to evaluate if cell age influences the response to dehydration. To determine the role of lipids during adaptation to desiccating conditions, the cultures were dehydrated for different periods and the FA of the cells were analysed. Regarding phenotypic modifications, the effect of dehydration on the net surface charge of the cells was also studied.

It is known that planktonic and biofilm cells have different degrees of tolerance to stress conditions, and for this reason it was also analysed if the biofilm cells have similar adaptation response to that presented by planktonic cells.

The production of storage lipids to assess if their presence could help the cells to survive under stress conditions was induced. The influence of these lipids on the tolerance of the cells to dehydration and to an antibiotic was studied.

## 3.1. Adaptation to dehydration

Most bacterial cells die when they are dehydrated, but some are able to withstand dehydration conditions. To study how *R. erythropolis* DCL14 cells are able to tolerate dehydration, the influence of some parameters on cell behaviour, and in particular, their response at the cellular membrane level, was determined. For that, cells were grown in planktonic state and as biofilm in media with different compositions and harvested at different times of cell growth. In all assays, for each sampling point following a certain dehydration period, the wet and dried weight of the samples was determined with the aim of verifying the dehydration level of the cells along time (**Figure 7**). This also allowed the validation of the method used.

The amount of water present in *R. erythropolis* samples decreased with dehydration time, as expected reaching 27% of dry weight of the cells after 180 min in the evaporator in both exponential and stationary cell phase in MM, and 30% and 23% for cells in exponential and stationary phases in MH, respectively (**Figure 7**). Larger amount of biomass was obtained in MH broth that in MM because of the nutrients available in the rich medium (**Figure 7 A, B *vs* C, D**). As expected, the amount of biomass collected in the stationary phase was higher than that harvested during the exponential phase (**Figure A, C *vs* B, D**).

Regarding the validation of the method, it was possible to observe that, in general, after 60 min the amount of water was maintained constant in the cells, indicating that these cells reached the maximum dryness achievable under the tested conditions (**Figure 7**).

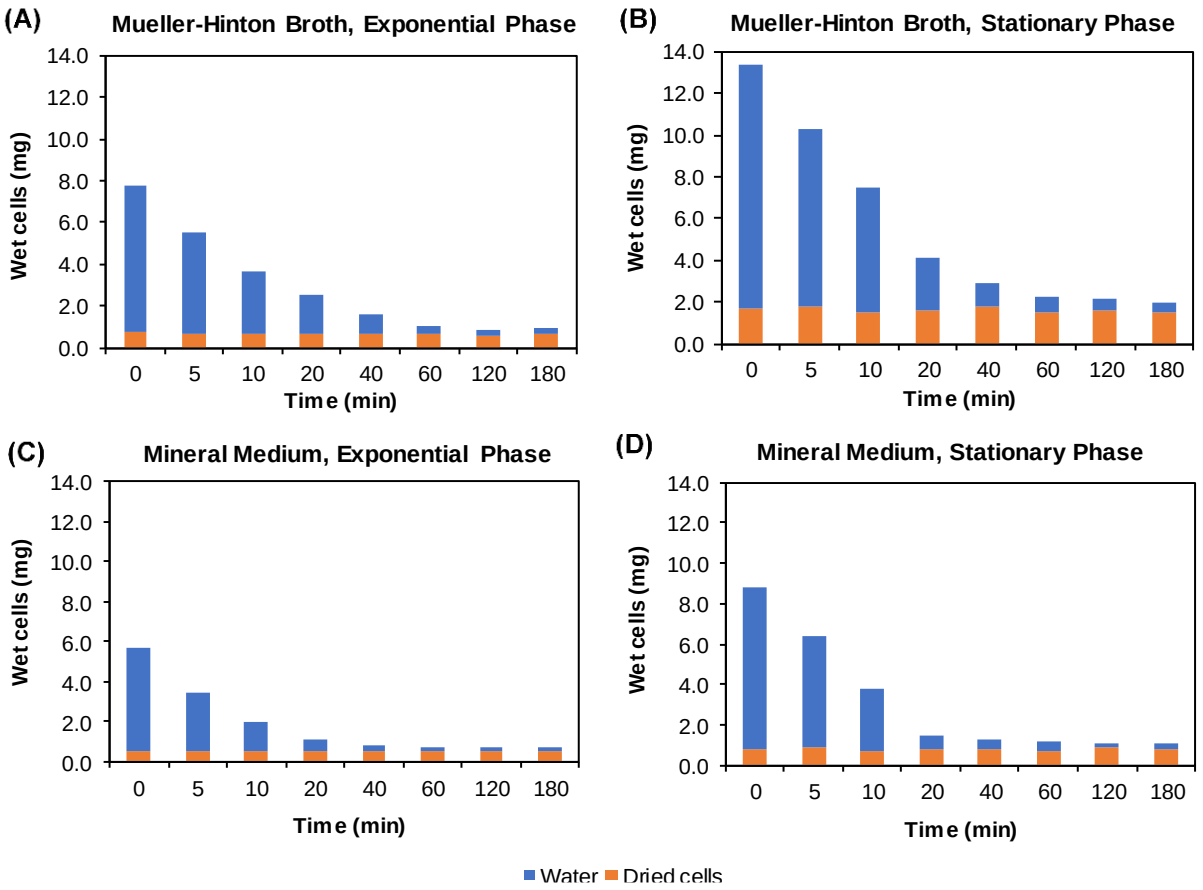


**Figure 7 –** Wet and dried weight of *R. erythropolis* DCL14 cells from cultures grown in different media and collected during the exponential and stationary phases, and respective amount of water present, after dehydration periods.

## 3.1.1. Influence of medium composition and cell growth phase on cell membrane

For this study, the cells of *R. erythropolis* DCL14 were collected from cell cultivation in planktonic state. This planktonic growth was promoted in two different liquid broth and analysed at different stages of cell growth: at exponential and stationary phases.

### 3.1.1.1. Fatty Acid Composition of the Cells

As mentioned in section 1.2 of Introduction, when bacteria suffer environmental stress, some bacteria use specific mechanisms that allow them to survive. Changes in the composition of the FA is one of the possible mechanisms of adaptation, and it is known that this alteration have an important role in bacterial adaptation to diverse stressful environmental conditions. Changes in structure and/or relative percentage of FAs of the phospholipids of the cellular membrane are made depending on the stress condition and are crucial to maintain the integrity and functionally of the membrane (de Carvalho 2012; Murinova and Dercova 2014; de Carvalho and Caramujo 2018).

The planktonic cultures were dehydrated for different periods and the FAs of the cells were analysed (**Figure 8**). To determine if the cells were still active and still able to change the membrane after dryness, the cells were rehydrated, and their FA profile analysed after 1h (**Figure 8**).

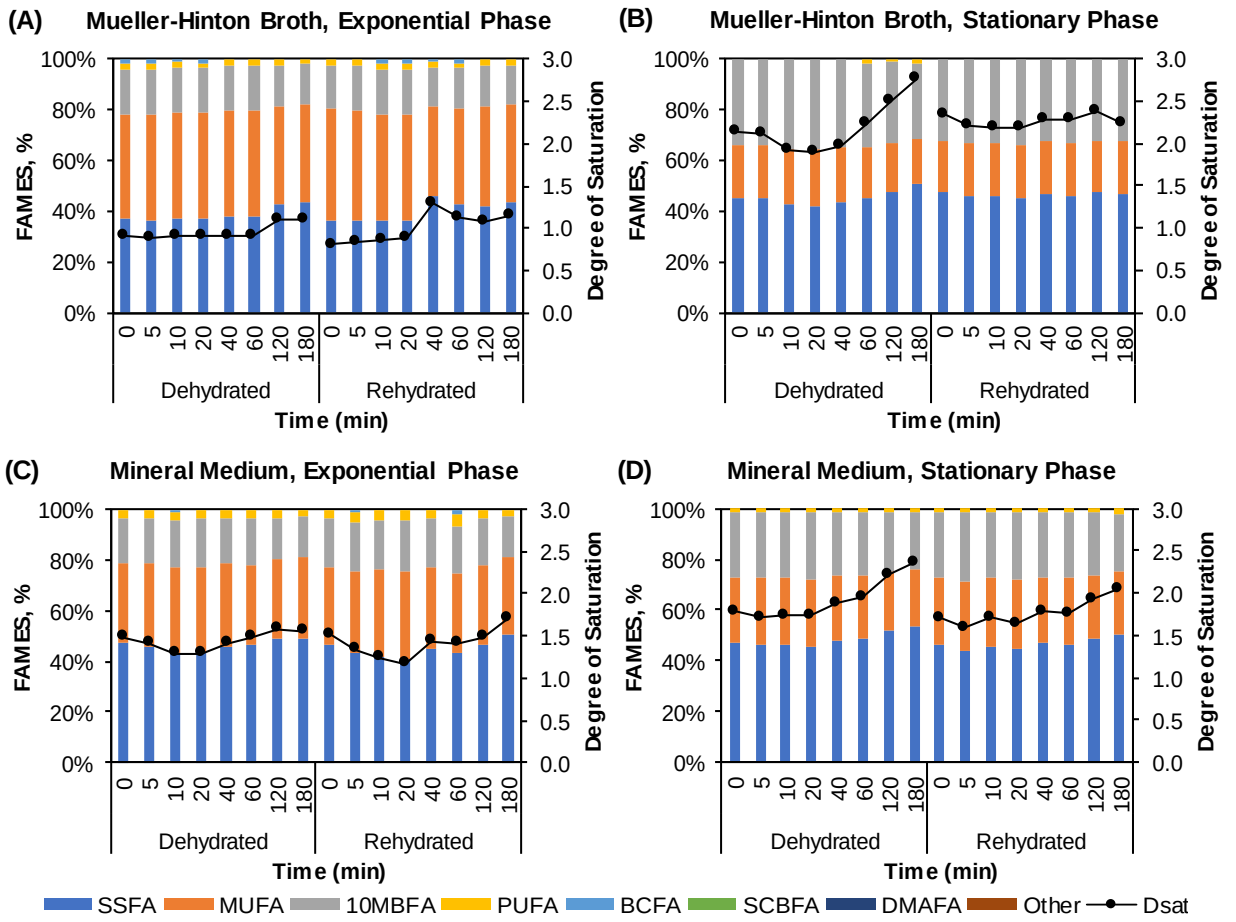


**Figure 8 –** Effect of growth media and dehydration time on the FA composition of *R. erythropolis* DCL14 cultures collected during different cell growth phases. The values represented are the mean of two independent assays. The line shows the corresponding degree of saturation of the FAs. **SSFAs**-saturated straight chain fatty acids, **MUFAs**-monounsaturated fatty acids, **10MBFAs**-10-methyl branched fatty acids, **PUFAs-**polyunsaturated fatty acids, **BCFAs-**branched-chain fatty acids, **SCBFAs-**saturated cyclopropyl-branched fatty acids, **DMAFAs-**dimethylacetals**, Other-**fatty acids that do not belong to the classes presented (only those presented at concentration higher than 1% are shown).

Through the analysis of **Figure 8,** it is possible to observe that the FA composition of cells is dependent on the growth medium and cell growth phase. However, the phospholipids of the cellular membrane of the cells grown on both cultivation media contained mainly SSFAs (at least 36.0%) and MUFAs (at least 18.0%). These FAs are the main regulators of membrane fluidity in the majority of bacterial strains (Russell, N.J. 1984). In gram-positive bacteria, such as *R. erythropolis*, 10MBFAs also occur at a considerable percentage (Denich, Beaudette *et al.* 2003). In the present study, *R. erythropolis* cells had a significant percentage of 10MBFAs, that varied between 16 and 35%, depending on the medium and age of the cells (MH, stationary phase was the condition that showed the highest value of 10MBFAs).

In both media, cells from control (t=0 min) decreased the percentage of PUFAs (MH: from 2.5 to 0.0%; MM: from 3.1 to 1.3%) and MUFAs (MH: from 41.4 to 21.1%; MM: from 28.9 to 24.8%), while increasing the percentage of 10MBFAs (MH: from 17.7 to 33.9%; MM: from 16.4 to 24.1%), when the cells reached the stationary phase (**Figure** 8). Relatively to the percentage of SSFAs, this was practically

the same regardless of the age of the cells in MM (from 44.7 to 44.1%) but increased in MH (from 37.0 to 45%).

In MH (**Figure 8A, B**), for the dehydrated cells the percentage of SSFAs was slightly different when the cells were in stationary phase (mean ± SD, 45.2 ± 2.6%) and exponential phase (38.8 ± 2.5%). However, the percentage of MUFAs was higher in exponential (40.9 ± 1.2%) than stationary phase cells (20.8 ± 1.4%), while the percentage of 10MBFAs was lower in exponential (17.4 ± 0.8%) than in stationary phase cells (33.6 ± 1.8%). For the rehydrated cells, the same behaviour was observed with slight differences in the percentage of SSFAs in exponential (40.0 ± 3.8%) and in stationary phase cells (46.5 ± 0.8%), higher percentage of MUFAs in exponential (40.3 ± 3.0%) than in stationary phase cells (20.6 ± 0.3%) and lower percentage of 10MBFAs in exponential (16.8 ± 0.8%) than stationary cells (32.9 ± 0.6%).

Analysing the exponential phase in MH (**Figure 8A**), no significant changes were observed in the lipid profile of the dehydrated cells when they were exposed from 0 to 60 min of dehydration, indicating the cells were not under stressful conditions due to the significant amount of water still present (**Figure 7A)**. However, an increase in the time of dehydration from 60 to 120 or 180 min, induced the cells to respond with an increase in the degree of saturation of FAs, thus decreasing the membrane permeability/fluidity. This increase in the degree of saturation was due to an increase in the percentage of SSFAs (from 37.4 ± 0.5% to 43.1 ± 0.4%) with a consequently decrease in the percentage of MUFAs (from 41.6 ± 0.2% to 38.9 ± 0.2%), 10MBFA (from 17.9 ± 0.2% to 16.0 ± 0.4%) and PUFAs (from 2.3 ± 0.2% to 2.0 ± 0.2%). The increased in the percentage of SSFAs was mostly due to the FA C18:0 (from 2.7 ± 0.1% to 10.0 ± 0.05%) (data not shown). The cells stopped producing BCFAs (more specifically the FA C11:0 iso) after 20 min of dehydration (from 1.3 ± 0.1% to 0%). In relation to the rehydrated cells, it was observed that for the times of dehydration from 0 to 20 min, the cells after 1 h of rehydration did not need to adjust again their FA profile, but the cells that were dehydrated for 40 and 60 min re-adjusted the membrane FA composition after rehydration. Their composition was different from the control cells. However, the cells exposed to 120 and 180 min of dehydrated conditions did not adjust the membrane FA composition, showing a profile similar to the dehydrated cells. The cells were probably unable to re-adjust their FA profile because of the long time they were dehydrated. This adjusts in FA profile resulted in variation in the degree of saturation.

Analysing now the stationary phase in MH (**Figure 8B**), only a slight decrease in the degree of saturation from 5 to 10 min (from 2.1 to 1.9), and a significant increase after 20 min in the evaporator in the dehydrated cells (from 1.9 to 2.8), were observed. Concomitantly to this increase in the percentage of SSFAs (from 43.6 ± 1.1% to 47.8 ± 2.2%; mainly due to the FA C18:0 that increased from 4.5 ± 1.1% to 7.3 ± 0.3%) (data not shown), the cells started producing PUFAs (more specifically the FA C18:3 ω6c from 0% to 1.3 ± 0.1%) (data not shown). The appearance of PUFAs was followed by a simultaneous decrease in the percentage of MUFAs. In relation to the rehydrated cells, no significant changes were observed between the control cells and those at other dehydration time.

In MM (**Figure 8C, D**), for the dehydrated cells in generally, the percentage of SSFAs and 10MBFAs was lower when the cells were collected during the exponential phase (SSFAs: 47.1 ± 1.0%; 10MBFAs: 17.9 ± 0.8%) than during the stationary phase (SSFAs: 49.1 ± 2.6%; 10MBFAs: 24.5 ± 1.4%).

The reverse behaviour was observed in the percentage of MUFAs and PUFAs, the exponentially grown cells (MUFAs: 31.8 ± 0.5%; PUFAs: 3.2 ± 0.1%) having higher percentage than cells collected during stationary phase (MUFAs: 25.1 ± 1.5%; PUFAs: 1.4 ± 0.2%). For the rehydrated cells, the same behaviour was observed in exponential (SSFAs: 45.7 ± 3.4%; 10MBFAs: 18.5 ± 1.4%; MUFAs: 32.0 ± 1.9%; PUFAs: 3.8 ± 0.4%) and stationary cells (SSFAs: 46.6 ± 2.0%; 10MBFAs: 25.8 ± 1.2%; MUFAs: 26.3 ± 0.9%; PUFAs: 1.4 ± 0.2%).

Analysing the lipid composition of the cells grown in MM and collected during the exponential phase, it is possible to observe a decrease in the degree of saturation from 0 to 20 min of dehydration (from 1.5 to 1.3) followed by an increase in the degree of saturation after 40 min in the evaporator (from 1.3 to 1.6). These changes in degree of saturation was due to the conversion of SSFA into MUFA or vice versa (**Figure 8C**). Besides the conversion in MUFAs, the SSFAs were also converted into 10MBFAs, as happened at 20 min of dehydration, where the highest value of this class is reached under these conditions (*c.a.* 19.78%). For the remaining classes, no significant alterations were observed. Regarding the rehydrated cells, a similar degree of saturation of FAs trend was observed (**Figure 8C**). Finally, analysing the lipid composition of the cells grown in MM and collected in the stationary phase, it is possible to observe for both dehydrated and rehydrated cells an increase in the degree of saturation of 1.33-fold and 1.19-fold, respectively, in comparison with the control cells (**Figure 8D**).

In dehydrated cells collected in the stationary phase, the cell membrane had more changes along dehydration periods than cells in the exponential phase, for both growth media (**Figure B, D**). Besides that, the degree of saturation of the FA of the stationary phase cells was on average 6.19% higher than that of control, while for the exponential phase cells was on average only 1.46% higher. These results indicate that the cells had a less fluid membrane as they became older.

In conclusion, significant differences in the FA composition of the cells were observed between the cells collected in the two growth phases (age of the cells) and between cells grown on the two growth media. However, the cell growth phase seems to have more impact in the FA composition than the growth media. Regarding the growth media, in a rich nutrient medium such as MH, the cells have nutrients at their disposal, while the opposite occurs in a poor nutrient medium such as MM. The availability of nutrients thus affects the membrane FA composition of the bacteria, since they can incorporate lipids that are available in the rich media, thus saving energy (Zhang, Y.-M. and Rock 2008). In fact, de Carvalho *et al.* presented a study where it was shown that *R. erythropolis* DCL14 adapt their FA composition as response to the carbon source used for growth (de Carvalho, Wick *et al.* 2009).

When exposed to dehydration, independently of growth media or growth phase, *R. erythropolis* DCL14 cells increased their degree of saturation which resulted in a decrease in the membrane permeability (**Figure 8**). This decrease in the membrane permeability preserves it in a liquid crystalline phase, maintaining its functionality and integrity, while avoiding the leakage of intracellular products and balancing the intracellular pH which is vital for the stability and activity of many intracellular proteins (Sinensky 1974; de Carvalho and Caramujo 2018). In relation to the rehydrated cells, not all the cells presented a profile similar to the control, indicating that the time for rehydration wasn't enough for the cells to recover, as was observed in the longer time of dryness, where the cells were totally dehydrated. Since the time of dehydration was longer, the cells were not able to adjust again their membrane FAs.

In most cases the changes observed in the profile are due to the conversion of SSFAs to MUFAs or PUFAs, or MUFAs to PUFAs by desaturases enzymes. The use of desaturase enzyme for the production of PUFAs was already reported in *Rhodococcus* strains (de Carvalho, Marques *et al.* 2014). In addition to these alterations, the conversion between MUFAs and BCFAs and between SSFAs and BCFAs were also observed, as reported by (Christie 1999).

### 3.1.1.2. Zeta Potential Analysis

The net surface charge of cells may be assessed by the zeta potential and is a physicochemical property that is related to the composition of the cell envelope and may affect the interactions and attachment in the process of cells adhesion and the entry of metabolites into the cells (Halder, Yadav *et al.* 2015). Bacterial surface charge, like other properties of the cells, are affected by environmental conditions such as temperature, pH or composition of the culture medium. However, under physiological conditions, they are negatively charged due to an excess of carboxyl and phosphate groups located in the cell wall (Zeraik and Nitschke 2012).

de Carvalho *et al.* showed that *R. erythropolis* DCL14 cells are able to adjust their surface net charge in response to environmental stresses or presence of toxic compounds (de Carvalho, Wick *et al.* 2009; de Carvalho 2012). In order to assess if there were some alterations in the surface charge of the cells along the dehydration time, the zeta potential of the cells was measured in samples collected simultaneously to those for the FAME analysis.

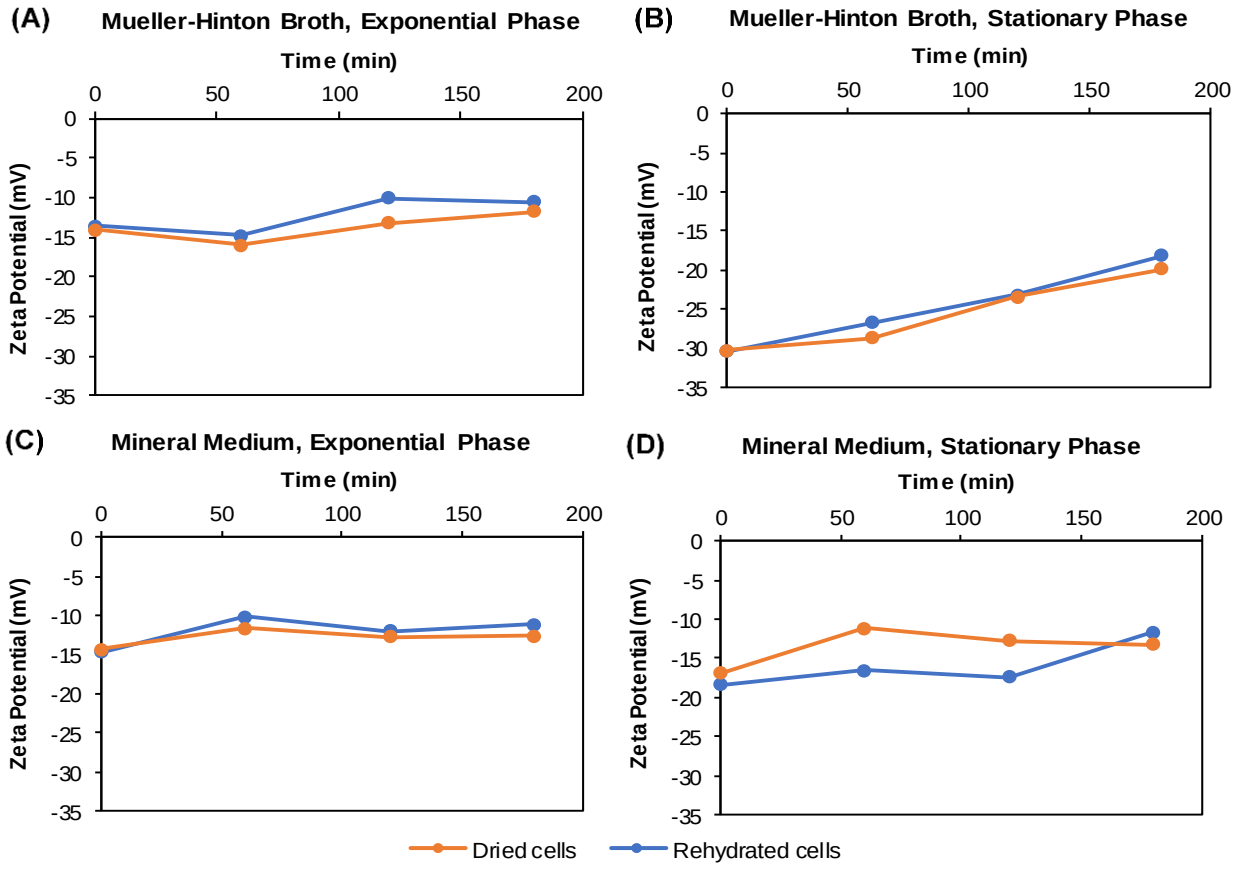


**Figure 9 –** Effect of dehydration time on the zeta potential of *R. erythropolis* DCL14 cultures grown on MH and MM media and collected during the exponential and stationary phases.

By comparing the net surface charge of the control cells, it is possible to observe that, regardless of the growth medium, the zeta potential of stationary cells was more negative than that of exponential cells ($MH_{exp}$ = -14.1 mV and $MH_{stat}$ = -30.3 mV; $MM_{exp}$ = -14.3 mV and $MM_{stat}$ = -16.9 mV) being this increased in negative charges more accentuated when the grown was performed in MH, probably due to a nonspecific adsorption of ions and molecules from the growth medium into the cell surface (**Figure 9**). The increase of negative charges when cells were in stationary phase was already observed for *Mycobacterium smegmatis* $mc^2155$ (Ayala-Torres, Hernández *et al.* 2014).

Along dehydration time, it is possible to observe that, independently of the growth phase and media, both dried and rehydrated cells become less negative and reached *ca.* -13.6 mV after 180 min. Curiously, dried and rehydrated cells presented similar values for the time of dryness/rehydration.

Although salinity stress and desiccation are different types stress, they are related because, they both cause a reduction of water activity, with repercussion in internal osmotic potential (Holzinger and Karsten 2013; Kumar, M., Kumari *et al.* 2014). Considering a study made by de Carvalho *et al.,* focused in the effect of increasing salt concentration in the cell envelope of *R. erythropolis* DCL14 cultures, an increase in the zeta potential values was observed with increasing time of exposure, independently of the concentration of salt (de Carvalho 2012; de Carvalho, Marques *et al.* 2014). The results obtained in the previous study showed results similar to those obtained for desiccated cells.

### 3.1.2. Effect of sodium chloride on lipid composition of the cells

As in the previous section, the planktonic cells of *R. erythropolis* DCL14 were collected from cell cultures. The planktonic growth was promoted in MM with and without salt and the lipid composition of the cells was analysed during exponential and stationary growth phases.

The planktonic cultures were dehydrated for different periods and the FAs of the cells were analysed with the aim of observing the influence of salt present in the medium on cell tolerance to desiccation (**Figure 10**). To determine if the cells were still active and still able to change the membrane after dryness, the cells were rehydrated, and their FA profile analysed after 1 h (**Figure 10**).

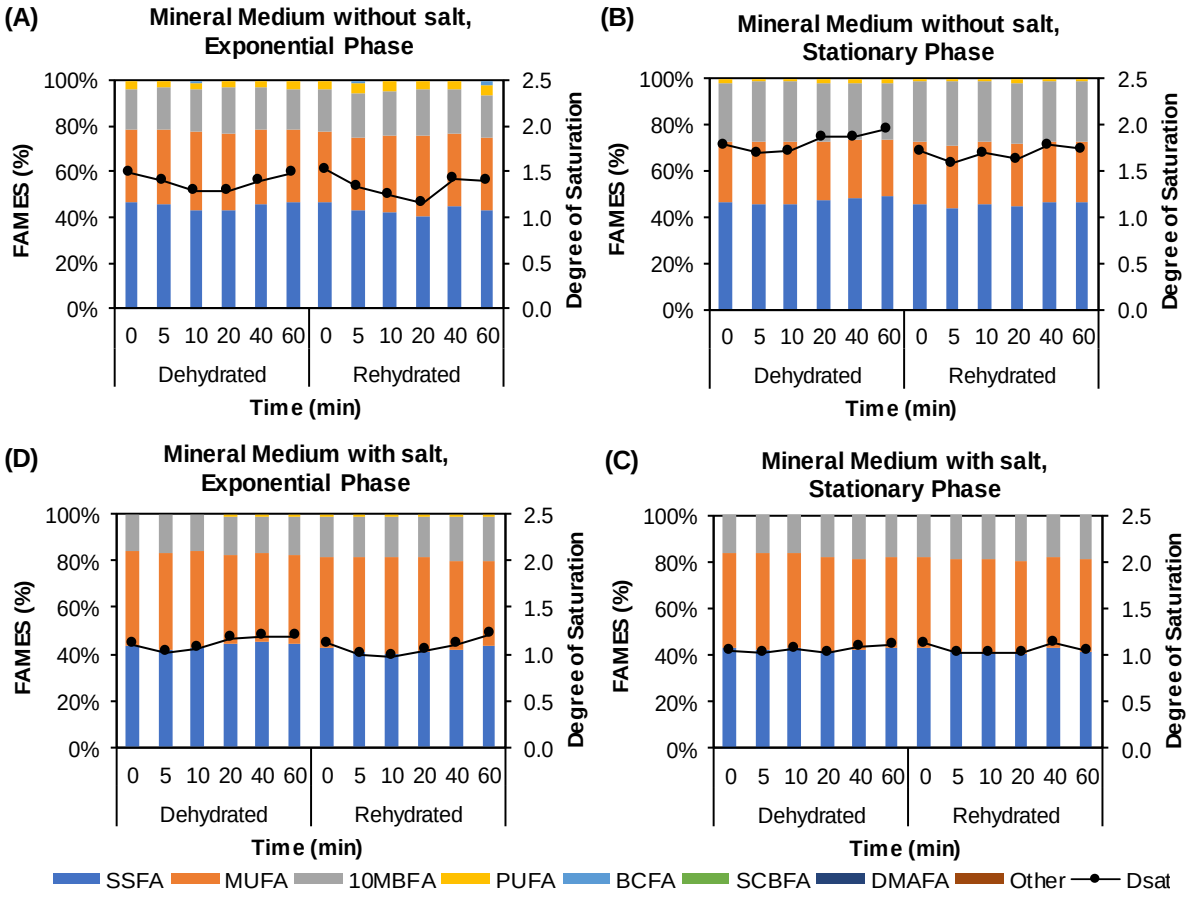


**Figure 10 –** Effect of salt concentration on growth media and dehydration time on FA composition of *R. erythropolis DCL14* cultures collected during different cell growth phases. The values represented are the mean of two independent assays. The line shows the corresponding degree of saturation of the FAs. The classification of each FA class is the same as that described in **Figure 8**.

Through the analysis of **Figure 10** it is possible to observe that the FA composition of cells, like in the previous study, is dependent of the growth medium even if the difference between the two is only the amount of salt. As in the previous study, for cells grown on MM with or without salt, the phospholipids of the cellular membrane contained mainly SSFAs (at least 40.0%), MUFAs (at least 24.0%) and 10MBFAs (at least 16%). Contrary to was observed for the cells grown in MM without salt, the cells grown in MM with salt did not show significant changes between both cell growth phases (**Figure 10**).

Analysing control cells, in both cell growth phases, it was observed that the cells grown in MM with salt showed a lower degree of saturation of the FA of the cells in comparison to those grown in MM without salt. A study performed by de Carvalho, in which the strain *R. erythropolis* DCL14 was exposed to an increasing concentration of sodium chloride, showed that at a concentration near to 35 g/L (concentration used in MM with salt), the cells had a lower degree of saturation of the FA in comparison to the cells grown without salt (de Carvalho 2012). These results were similar to those obtained in this study.

Analysing the lipid composition of the cells grown in MM and collected during the exponential phase, it is possible to observe a decrease followed by an increase in the degree of saturation due to the conversion of SSFA into MUFA or vice versa. For the cells that were dehydrated during 20 min, there was also a conversion of SSFAs in 10MBFAs. Regarding the rehydrated cells, a similar degree of saturation of FAs trend was observed (**Figure 10A**). Finally, analysing the cells collected during the stationary phase in MM without salt, it is possible to observe, for both dehydrated and rehydrated cells, an increase in the degree of saturation of 1.10-fold and 1.02-fold, in comparison with the control cells (**Figure 10B**) (for a more detailed analysis see section 3.1.1.1).

Comparing FA profile obtained for the cells that grew in MM with and without salt, it was possible observed that the cells that grew in MM without salt responded more to dehydration than the cells that grew in MM with salt. These slight differences observed in the cells that grew in MM with salt were probably due to the stress induced by dehydration and the presence of salt, indicating that the presence of salt influence the response of the cells to dehydration. However, analysing the exponential phase in MM with salt (**Figure 10C**), it was observed a slight increase in the degree of saturation (from 1.06 ± 0.03% to 1.16) after 20 min of dehydration. This increase was mostly due to an increase in the percentage of the FA C18:0 (from 6.9 ± 1.1% to 10.9 ± 0.6%), with a concomitant decrease in the percentage of MUFAs (data not shown). This decrease in the percentage of MUFAs was due to a conversion in SSFAs but also to a conversion in PUFAs (more specifically the FA C18:3 ω6c from 0% to 1.1 ± 0.1%) (data not shown). In relation to rehydrated cells no significant changes were observed between the control and those collected at other dehydration times. To finish, analysing the lipid composition of the cells grown in MM with salt and collect in the stationary phase, no significant changes were observed in the lipid profile of the dehydrated and rehydrated cells, which indicates that the cells would not or could not respond to these conditions (**Figure 10D**).

Regarding to the composition of the growth media, it was observed that the influence of the availability of nutrients, evaluated in the previous section, had more relevance than the presence of salt in the FA composition of the cells. In the present study, it was observed that the presence of salt in the growth medium decreased the degree of saturation of the FA of the membrane. This behaviour could be a response by the cells for the stress induced by dehydration and presence of salt.

### 3.1.3. Effect of mode of growth (planktonic *vs* biofilm) on cell tolerance to dehydration

The planktonic cells of *R. erythropolis* DCL14 were collected from a cell suspension growing in MM with or without salt, while the biofilm growth was promoted on 24-well plates for 24 h in MM with or without salt. Planktonic and biofilm cells showed some differences in relation to the mode of growth, cell physiology and stress tolerance, being their properties strictly related with the culture conditions.

In the biofilm growth mode, the cells, in general, are enclosed in a self-produced extracellular matrix that consists predominantly of an EPS consisting of polysaccharides, proteins, lipids, extracellular DNA (eDNA) (Flemming and Wingender 2010). This EPS matrix is highly hydrated due to the large percentage of water (about 95%) that are incorporated into its structure by hydrogen bonding and, since it dries slower than its surroundings, because their hygroscopic nature, it protects the biofilm cells against fluctuations in water content (Roberson and Firestone 1992).

Regarding the present study, the FA profile of both planktonic and biofilm cultures were dehydrated for different periods and the FAs of the cells were analysed with the aim of observing the influence of the growth mode on tolerance to desiccation (**Figure 11**). To determine if the cells were still active and still able to change the membrane after dryness, the cells were rehydrated, and their FA profile analysed after 1 h (**Figure 11**).

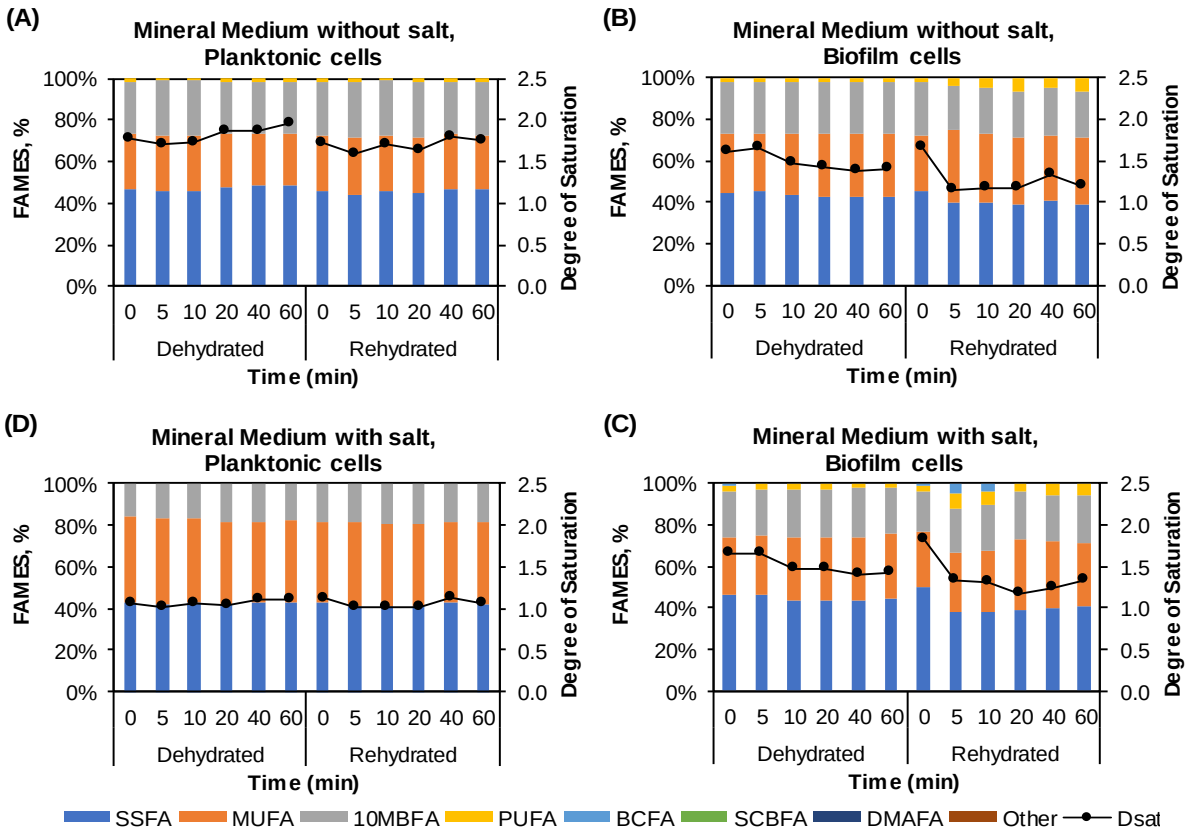


**Figure 11 –** Effect of dehydration time on FA composition of *R. erythropolis DCL14* cultures grown in different media in planktonic and biofilm modes. The values represented are the mean of two independent assays. The line shows the corresponding degree of saturation. The classification of each fatty acid class is the same as that described in **Figure 8**.

Through the analysis of **Figure 11,** it is possible to observe that the FA composition of cells is dependent on the mode of growth, as planktonic and biofilm cultures. However, as in previous studies, the phospholipids of the cellular membrane of the cells, contained mainly SSFAs (at least 38.0%), MUFAs (at least 24.0%), and 10MBFAs (at least 16.0%). Analysing, cells from control in the presence and absence of salt, it was observed that the biofilm cells had a FA profile more similar to each other than planktonic cells (**Figure 11**). Biofilm cells produced 2% more MUFAs and 1% more PUFAs than planktonic cells in MM without salt, but the reverse was observed to SSFAs and 10MBFAs, respectively. In relation to MM with salt, biofilm cells produced more SSFAs (3%), 10MBFAs (6%), PUFAs (3%) and BCFA (1%) than planktonic cells, but much less MUFAs (13%).

Considering now the lipid composition of the cells grown in MM without salt, it is possible to observe a lower degree of saturation of FA in biofilm cells than in planktonic cells. The same behaviour was demonstrated by de Carvalho in other studies for the same bacteria grown as planktonic or biofilm (de Carvalho, Wick *et al.* 2009; Rodrigues and de Carvalho 2015). Contrary to that observed for MM without salt, in MM with salt the cells present a higher degree of saturation of FA of cells when grown as biofilm. The higher degree of saturation of FA for biofilm cells in comparison with planktonic cells was also observed for *Pseudomonas putida* mt-2 grown as planktonic and biofilm cells in the presence of salt, by Hachicho *et al.* (Hachicho, Birnbaum *et al.* 2017).

No significant changes were observed in the FA profile of biofilm cells grown in MM without salt along the dehydration time, for the dehydrated cells, between the control and 5 min of dehydration (**Figure 11B**). However, an increase in the time of dehydration from 10 to 60 min, induced the cells to respond with a decrease in the degree of saturation (from 1.62 ± 0.02 to 1.42 ± 0.04). This decrease was mainly due to a decrease in the percentage of the FA C18:0 (from 12.9 ± 0.2% to 10.0 ± 0.6%) with a concomitantly increase in the percentage of MUFAs (from 27.9 ± 0.3% to 30.0 ± 0.4%) (data not shown). For the remaining classes, no significant changes were observed. Regarding the rehydrated cells, it was observed a decrease in the degree of saturation (from 1.68 to 1.20 ± 0.07) from control to 5 min of dehydration followed by a constant value (**Figure 11B)**. This decrease was mainly due to a decrease in the percentage of the FA C16:0 (from 28.6% to 23.8% ± 0.7), with a simultaneously decrease in the percentage of 10MBFAs (from 26.0% to 22.0 ± 0.8%) and an increase in the percentage of unsaturated FA (MUFAs from 26.8% to 33.0 ± 1.4% and PUFAs from 2.0% to 5.4 ± 0.9%). The changes observed in PUFAs and 10MBFAs were mainly due to the FA C18:3 ω6c and C18:0 10-methyl (data not shown). Concerning the lipid composition of the biofilm cells grown in MM with salt, it was observed a behaviour similar to the biofilm cells grown in MM without salt, for both dehydrated and rehydrated cells (**Figure 11D**). However, in rehydrated biofilm cells grown in MM with salt, it was observed a lower percentage of BCFA (due to FA 11:0 iso and 13:0 anteiso) after 5 and 10 min of dehydration due to a conversion of MUFAs (data not shown)

Unlike in previous studies for planktonic cells, the biofilm cells had a decrease in the degree of saturation along the dehydration conditions. Such behaviour can be explained by the fact that they were in a hydrated environment, increasing the permeability of the membrane. When exposed to rehydration, the cells showed a similar behaviour with to dehydrated cells but different from the control.

### 3.1.4. Cell Viability Analysis

The morphology and viability of *R. erythropolis* DCL14 cells exposed to dehydration were analysed by fluorescence microscopy with the objective to visualize viable cells stained green (cells with an intact membrane) and non-viable cells stained red (cells with a damaged membrane), along dehydration time.

The cell viability was determined for cells grown in different growth media and collected during the cell growth phase, for the following dehydration times: 0, 40, 60, 120 and 180 min. For that, the number of viable cells was counted and used to determine the respective percentage. This parameter allows the evaluation of the tolerance of this bacterium to dehydration and the effect of the cell growth phase and the growth media on this tolerance (**Figure 12**).

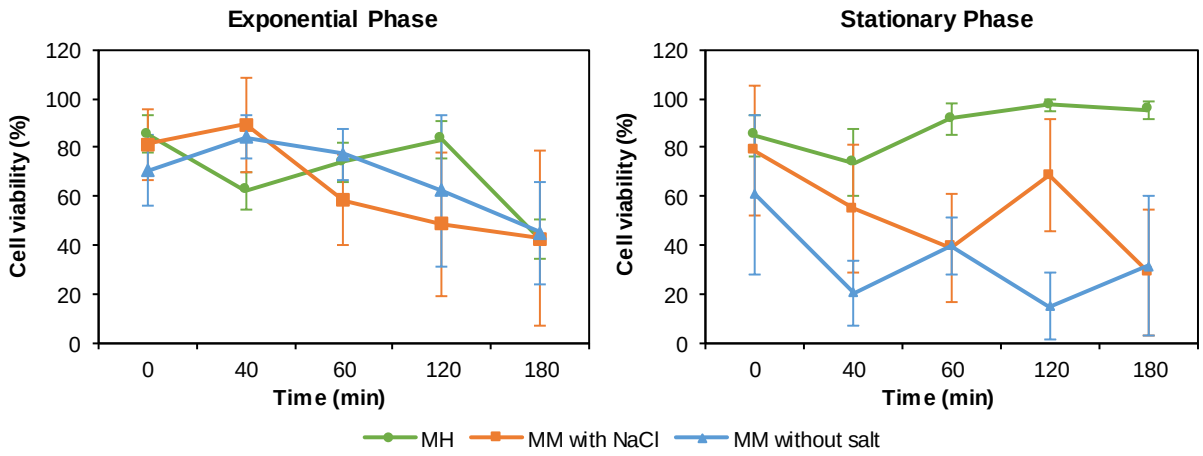


**Figure 12 –** Percentage of viable cells of *R. erythropolis* DCL14 cells along dehydration time. Error bars represent the respective standard deviation.

The percentage of viability calculated through the number of viable cells in the total population of *R. erythropolis* DCL14, decrease with the dehydration time **(Figure 12).** Analysing the exponential phase cells, it was observed that regardless of the composition of the growth medium, the viability values reached a similar value after 180 min of dehydration (MH: 42.4%; MM with salt: 42.6%; MM without salt: 44.9%). Regarding the stationary phase cells, contrarily to what was observed in exponential phase, there were differences in the control cells depending on growth media. The lower percentage was obtained for the cells grown in MM with salt. However, after 180 min of dehydration the cells grown in MM with and without salt showed a similar value (MM with salt: 28.8%: MM without salt: 31.4%) but lower in comparison with the cells grown in MH (95.4%).

In conclusion, it was observed that when the cells were grown in MH the percentage of viable cells was higher in stationary phase cells than in exponential phase cells. However, for MM with and without salt, this difference was not observed.

## 3.2. Influence of storage lipids on cell adaptation

Cell cultures in late stationary phase are known to be more resistance to stressful conditions (Kolter, Siegele *et al.* 1993; McLeod and Spector 1996; Kimura, Ookubo *et al.* 1997). In the present study, it was hypothesized that the increase tolerance could be partially ascribed to lipids droplets containing storage lipids. The accumulation of lipids was thus induced in *R. erythropolis* DCL14 cells through the addition of an excess of carbon source to the culture media. After this lipid accumulation, the cells were dehydrated during 180 min and exposure to an antibiotic, with the objective to verify if the bacterium became more tolerant/resistant.

### 3.2.1. Cell response to dehydration

The strains from the species *R. erythropolis* are known to use a varied range of carbon source due to their metabolic diversity and versatility. The accumulation of specialised lipids, such as TAGs, has been associated with the stationary growth phase or when the amount of carbon source was higher than the nitrogen source (Alvarez, H.M., Silva *et al.* 2013; Cortes and de Carvalho 2015). To determine if the two sugars and their concentration influence the FA profile of the cells, the cells were analysed, firstly, under non-stress conditions (**Figure 13**) and secondly under stress conditions (**Figure 14**).

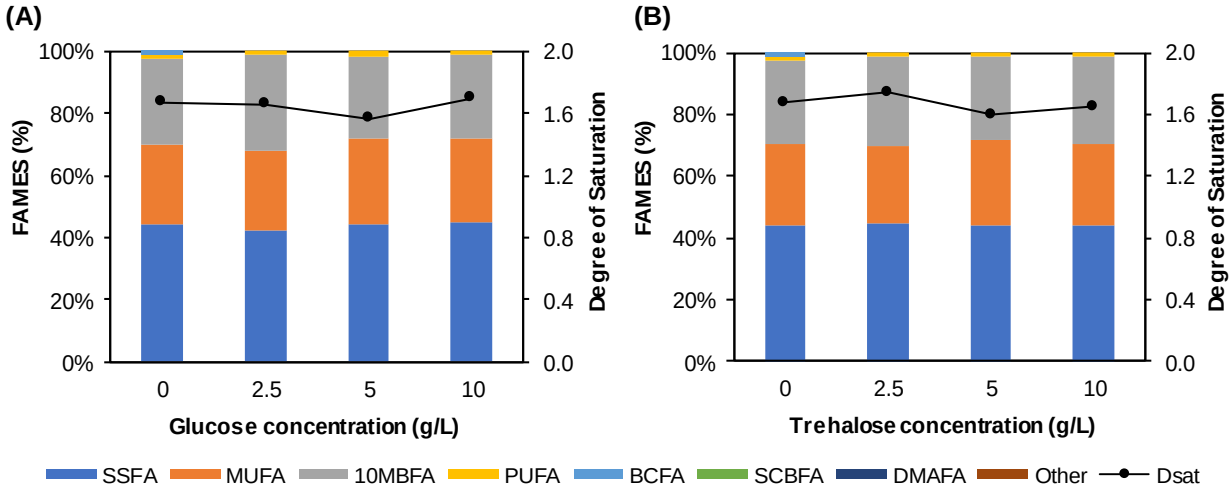


**Figure 13 –** Effect of sugar and their concentration on fatty acid profile of *R. erythropolis DCL14* cultures. The values represented are the mean of two independent assays. The line shows the corresponding degree of saturation. The classification of each fatty acid class is the same as that described in **Figure 8**.

Through the analysis of **Figure 13**, it is possible to observe that there were not significant changes in membrane FA composition of the cells that were grown in the presence of different sugars and concentrations, probably due to lower concentrations used. Regardless of the sugar and concentration, the phospholipids of the cellular membrane of the cells contained mainly SSFAs (at least 40.0%), MUFAs (at least 24.0%), and 10MBFAs (at least 24.0%), as verified in section 3.1. The only significant alteration was in the BCFA class, because only control cells (no sugar addition) had this class. Verifying that both sugars used and their concentrations, under non-stress conditions, did not influence the FA profile of *R. erythropolis* DCL14, it was also relevant to study their influence under stress conditions (**Figure 14**).

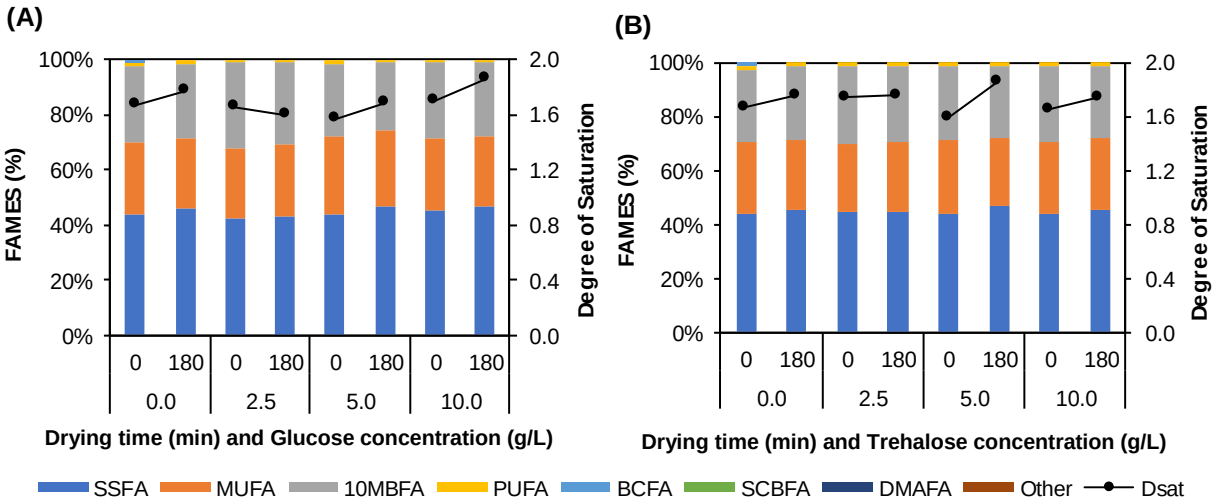


**Figure 14 –** Effect of the type sugar and concentration on dehydration-resistance at the level of the composition of the fatty acid, on *R. erythropolis DCL14* cultures. The values represented are the mean of two independent assays. The line shows the corresponding degree of saturation. The classification of each fatty acid class is the same as that described in **Figure 8.**

Analysing **Figure 14**, it is possible to observe that independently of the sugar and concentration, the behaviour of the cells after 180 min of drying is similar between them and with that observed in the dehydration assays in section 3.1. Such as in section 3.1, the cells decreased the fluidity of membrane as response to dehydration, showing a FA profile similar to that obtained for cells grown in MM without salt and harvested during stationary phase (section 3.1.1.1).

This increase in degree of saturation was due to a slightly increase in the percentage of saturated in relation to the percentage of unsaturated FA, more specifically to the classes SSFAs and MUFAs. For the concentration of 2.5 g/L of both sugars, it was observed an opposite behaviour: there was an increase in the percentage of unsaturated in relation to the saturated FA. However, the observed behaviour allowed the resistance of the bacteria cells to dehydration. In conclusion, with or without lipid accumulation, the behaviour of the cells as response to dehydration was the same.

### 3.2.2. Cell response to antibiotic exposure

When exposed to antibiotics, bacterial cultures can acquire a resistant, tolerant or persister behaviour. The term “resistance” is used when microorganisms have an inherited ability to grow at high concentrations of an antibiotic, whereas the term “tolerance” is used to describe microorganisms that have the ability to survive transient exposure to a high concentration of an antibiotic (Scholar and Pratt 2000). In contrast with these two terms that are related with whole bacterial populations, the term “persistence” is a transiently phenotypic tolerance of a subpopulation of a bacterial population when expose to a high concentration of an antibiotic (Lewis 2007; Gefen and Balaban 2009). This subpopulation can be easily detected through a biphasic pattern in killing curves when monitoring bacterial populations exposed to an antibiotic (Balaban, Merrin *et al.* 2004).

Resistance is associated to numerous molecular mechanism such as target modification by mutation or specialized enzymatic changes, target substitution, antibiotic efflux, antibiotic modification

or destruction (Lewis, Salyers *et al.* 2002; Levy and Marshall 2004). All these mechanisms have the same objective: to avoid the binding of the antibiotic to its target. In contrast, persistent tolerance is generally explained by a transition to a dormant state, which is characterized by a reversible reduction of metabolism and growth rate, such as little or no cell wall synthesis, translation or topoisomerase activity (Lewis 2010). Besides this dormant state, persistent cell tolerance may be due to antibiotic target blockade through the action of “persisters proteins” (Lewis 2007). Even if the antibiotic is bound to the target, it will be unable to corrupt its function, because an active target is needed in order for an antibiotic to act and cause the death.

In conclusion, when a bacterial population is exposed to a high concentration of a bactericidal antibiotic, regular cells die while persisters survive. Therefore, it was important to determine the maximum concentration tolerated by the cells, considering both sugars and concentrations, to evaluate if cells with accumulated lipids were more resistant than cells without lipid accumulation. For that, the culture was exposed to a certain concentration chosen through MIC values and the cells samples were collected and plated for CFU counting over the time.

### 3.2.2.1. Determination of the MIC

With the objective to select the maximum concentration of vancomycin tolerated by the *R. erythropolis* DCL14 cells, grown in different sugars and concentrations, the MICs was determined. In **Table 4** are shown the MICs obtained, and it is shown an example image for the determination of MIC.

**Table 4 –** Values of MIC obtained for each concentration of sugar.

| **Sugar (g/L)** | | **MIC (**µg/mL) |
|---|---|---|
| 0 | | 4 |
| Glucose | 2.5 | 4 |
| | 5.0 | 2 |
| | 10.0 | 1 |
| Trehalose | 2.5 | 4 |
| | 5.0 | 1 |
| | 10.0 | 1 |

Through the analysis of **Table 4** is possible to observe that the MIC values of the cells that grew in presence of both sugars were similar. It is also observed that with the increasing sugar concentration there were a decrease in the MIC value. Based on the MIC values obtained, a concentration of vancomycin of 2 µg / mL was chosen for the following analyses.

### 3.2.2.2. Time-Dependent Killing

In **Figure 15** it was shown the susceptibility of exponential growing *R. erythropolis* DCL14 cultures to vancomycin **(Figure A.1)**, which is a bactericidal glycopeptide. This glycopeptide, discovered in 1953, interferes with the cell wall biosynthesis, because it binds to the D-Ala-D-Ala dipeptide terminus of the peptidoglycan cell-wall precursor via the formation of five hydrogen bonds. This complex prevents the transglycosylation and transpeptidation reaction but also the incorporation of the precursors into the bacterial cell wall (Arthur, Reynolds *et al.* 1996; Walsh 2003). Thus, to study the effect of the lipid accumulation in the tolerance/resistance of *R. erythropolis* DCL14 cells towards vancomycin, they were collected and exposed for a total of 5 h to a concentration of 2 µg/mL of vancomycin. The CFU numbers, after different time of exposure, were determined and used to determine the percentage of survived cells (**Figure 15).**

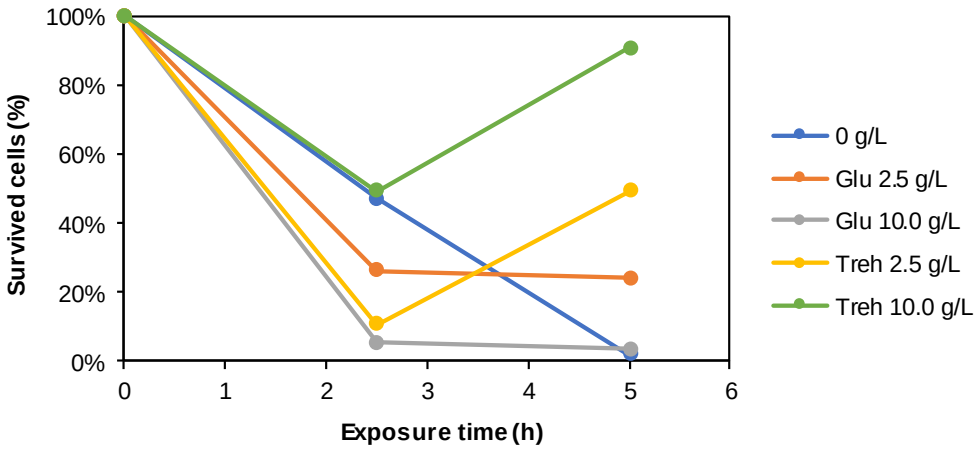


**Figure 15 –** Percentage of *R. erythropolis* DCL14 that survive during 5 h of exposure to 2 µg/mL of vancomycin in different concentration of glucose (Glu) and trehalose (Treh) as carbon source.

It is possible to observe in **Figure 15**, the typical biphasic killing patterns showed by Lewis (Lewis 2007) where a distinct plateau of surviving persisters cells were represented by cells that grew in glucose. Cells that grew in trehalose and exposed to vancomycin for 5 h presented increased survival when compared to cells collected after 2.5 h, indicating that these cells presented resistance to vancomycin. Generally, the formation/isolation of persister cells is increased upon exposure to high doses (high MIC equivalents) of antibiotics (Moyed and Bertrand 1983). However, for cells with lipid accumulation it was not necessary a high dose for the appearance of persister cells. In fact, the concentration used was lower than the minimal inhibitory concentration determined.

A possible explanation to the resistant cells observed, is the accumulation of vancomycin inside the lipid droplets (LDs) that were accumulated through the excess of carbon (promoted by the addition of the sugar) and the limitation of nitrogen. This accumulation of vancomycin inside the LDs could contribute for a protection under antibiotic exposure to the bacterial cells. Beside this protection the LDs may participate in some cell functions, such as phospholipid recycling, cell signalling, membrane trafficking, metabolism of intracellular proteins (Zhang, C., Yang *et al.* 2017; de Carvalho and Caramujo 2018). In *Mycobacterium tuberculosis* it was shown that accumulation of lipid droplets is associated with antibiotic tolerance and persistence (Daniel, Maamar *et al.* 2011; Vijay, Hai *et al.* 2018).

### 3.2.2.3. Fatty Acid Composition Analysis

In order to study the adaptation mechanisms of *R. erythropolis* DCL14 during different times of exposure to vancomycin and evaluated the influence of lipid accumulation in that adaptation, cells were collected at specific times during the time-dependent killing curve (**Figure 15**) and it was analysed the variations at the level of the composition of the FA (**Figure 16**).

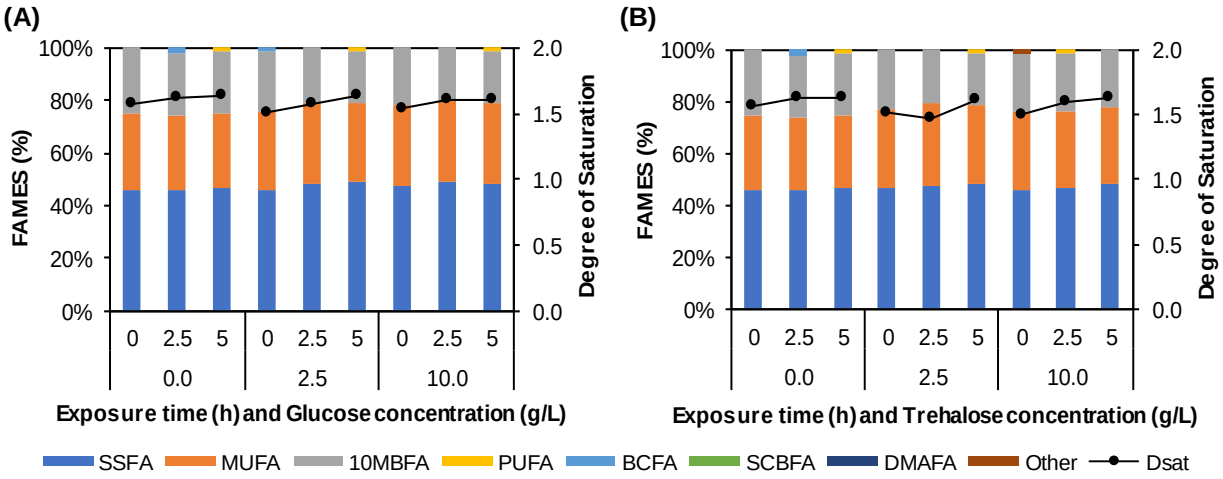


**Figure 16 –** Effect of the type of sugar and concentration on antibiotic-resistance during the time of exposure at the level of the composition of the fatty acids, on *R. erythropolis* DCL14 cultures. The line shows the corresponding degree of saturation. The classification of each fatty acid class is the same as that described in **Figure 8**.

Through analysis of **Figure 16**, it was observed that regardless of the type of sugar and concentration, *R. erythropolis* DCL14 cells slightly increased the percentage of SSFAs while increasing the percentage of MUFAs, and decreasing the percentage of 10MBFA, along the time of exposure to vancomycin. This increase in the percentage of SSFAs was mostly due to the FAs C12:0 and C18:0 (data not shown). Most of the alterations were observed during the first 2.5 h of exposure, indicating a rapid mechanism of response by the bacterial cells. Both sugars and their concentrations showed the appearance of a low concentration (1-2%) of PUFAs (more specifically the FA C18:3 ω6c; data not shown) after 5 h of exposure, except at a concentration of 10 g/L of trehalose, where this class of FA appears at 2.5 h instead 5 h of exposure. This appearance was due to the conversion of SSFA or MUFA in PUFA, through the action of desaturases.

During the exposure to the antibiotic, *R. erythropolis* DCL14 responded with a slight increase in the degree of saturation, in almost all conditions. These results suggest that the bacterium respond to the stress induced by vancomycin, with a decrease in membrane fluidity. This decrease in membrane fluidity was in agreement with those published in the literature for the presence of toxic organic compounds or other extreme environmental conditions (de Carvalho 2010;2012; Murinova and Dercova 2014).

# 4. Conclusions and Future Work

The bacterium *Rhodococcus erythropolis* is extremely adaptable and tolerant to several toxic compounds and stressful conditions, and therefore may play a crucial role in biotechnology. This tolerance is associated to its metabolic diversity and membrane properties, where modifications in lipid composition of the cellular membrane are one of the most important adaptation mechanisms of this bacterium.

In the present work, the physicochemical properties of the surface and the lipid composition of the membrane of *R. erythropolis* DCL14 cells when exposed to stressful conditions, such as dehydration and exposure to antibiotics, were studied.

The first study aimed to verify the tolerance of this bacterium to dehydration conditions, as well as to verify if the growth conditions were a determinant factor in the tolerance. It was observed that the FA profile is dependent on the composition of the growth medium, but also on the age of the cells at the time of harvest and the mode of growth (planktonic or in biofilm). For the cells grown in planktonic state, for the three media used, a decrease in the percentage of MUFAs and an increase in the percentage of SSFAs were observed along the dehydration time, which indicates that the cell adjusted their membrane composition to reduce the fluidity of the cellular membrane and consequently preventing the leakage of intracellular compounds. In most of the cases, this increase in the degree of saturation was due to the direct conversion of MUFAs in SSFAs. Besides this direct conversion, it was also observed a decrease in the MUFAs through the conversation to PUFAs or through the conversion into BCFAs. The increase observed in the degree of saturation of the membrane was related, in almost all conditions, with the increased percentage of the FA C18:0 along the dehydration time. Comparing to what was observed for the planktonic cells, biofilm cells decreased the degree of saturation, which resulted in a more fluid membrane.

During cellular adaptation to dehydration, cells changed their FA composition in order to achieve the best membrane fluidity that allowed the survival, and a FA that seems strongly correlated with the adaptation to dehydration is the FA C18:0 since its percentages was been greatly affected by the time of dehydration. Besides, the modifications on FA composition during cellular adaptation it was verified that along the dehydration the cells were able to change their zeta potential. Regardless of the conditions (cell age at the moment of harvest and growth composition), the cells become less negative and reached *ca.* -13.6 mV after 180 min of dehydration, although they had different initial values.

To conclude this study, cell viability was also evaluated in order to observe the effect of dehydration on the global population. A decrease in the viability of the cells was observed along the time for both media analysed. However, after 180 min a high percentage of viability was still observed, indicating that this bacterium is extremely tolerant to the conditions of dehydration.

The promoted lipid accumulation aimed at assessing if there they could influence tolerance to dehydration and exposure to antibiotics, when the cells accumulated storage lipids. The cells exposed to dehydration did not show significant modifications on FA profile with or without accumulation of lipids. The FA profiles obtained here were similar to those observed in the first study and the same behaviour was also observed. In relation to the cells that were exposed to vancomycin a biphasic killing pattern was observed after 2.5 h of exposure: the cells that grew in glucose showed a persister behaviour; the

cells that grew in trehalose showed a resistant behaviour. These results indicated that accumulation of vancomycin inside the LDs could increase the resistance of the *R. erythropolis* DCL14 cells to vancomycin. Through the analysis of the FA profile, it was observed that the cells, in response to the presence of the antibiotic, increased the degree of saturation, thus leading to a more rigid membrane. The observed response at the lipid level for the presence of vancomycin was the same observed when the bacterial cells were exposed to several toxic compounds.

The results obtained in this work, enriched the knowledge about the high tolerance and adaptability of this bacterium to challenging conditions. This work served, principally, to promote a future in-depth study of bacterial dehydration in order to better understand the mechanisms used, the consequences for the bacterial cells, and what are the desiccation limits. Relative to the exposure to antibiotics, further studies are required for a better clarification. Thus, the following studies are suggested:

1. Study the influence of the cell wall composition (*e.g.* MA) during dehydration;
2. Study the composition of the EPS and the relationship between its quantity and dehydration time;
3. Determine the maximum exposure time that the cells endure until they all die;
4. Evaluate the influence of dehydration at enzymatic level;
5. Evaluate the tolerance/resistance of the bacterium exposed to a high concentration of vancomycin or to other antibiotics, after lipid accumulation.

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# Appendix

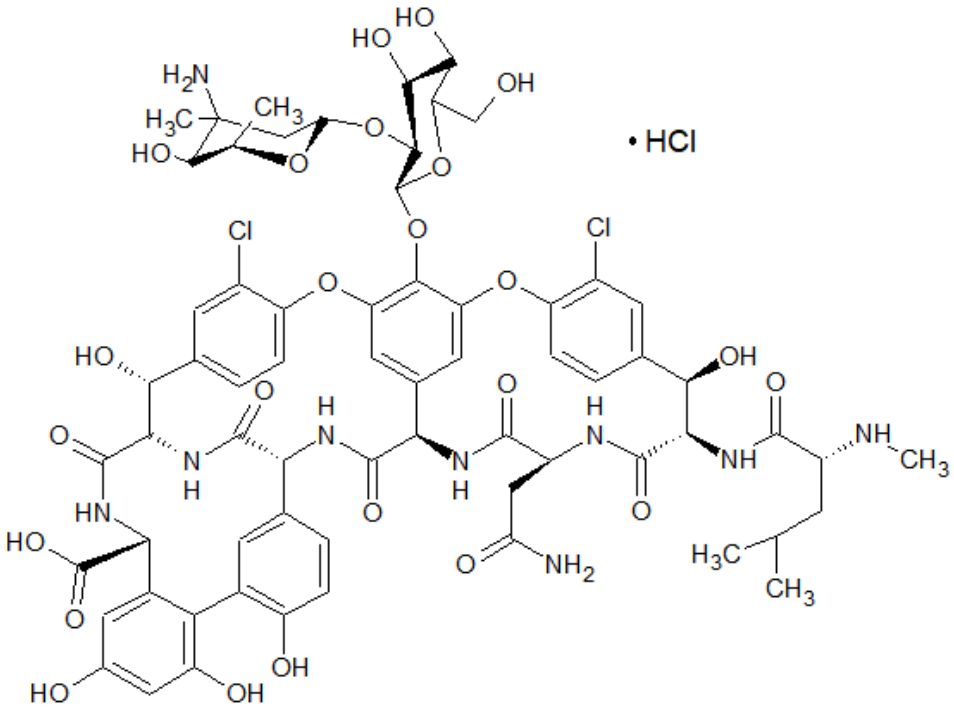


**Figure A. 1 –** Chemical structure of the glycopeptide vancomycin.