Understanding *Rhodococcus erythropolis* adaptation to extreme conditions

Catarina Coelho Dias
catarinacdias@tecnico.ulisboa.pt

Instituto Superior Técnico, Lisboa, Portugal

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Abstract

Bacterial cells are exposed to several environmental conditions, and they use various adaptive mechanisms to survive. Several strains of the genus *Rhodococcus* have been studied for the understanding of their metabolic pathways and the adaptive mechanisms responsible for its adaptability to diverse conditions. 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 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. Lastly, 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 a reduce in membrane permeability, 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

1. Introduction

Organisms can be found in diverse environments, including extreme conditions (*e.g.* temperature, pressure, desiccation, salinity, pH), and 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 industrial processes (Morozkina, Slutskaya *et al.* 2010).

Extremophiles organisms may be classified in extremophilic or extremotolerant, and the difference between both is that the first require one or more extreme condition to grow while the second can tolerate one or more extreme condition but grow optimally under moderate (Rampelotto 2013). Extremophilic organisms have particular characteristics that enable their ability to inhabit extreme environments 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 (Rhothschild 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 desire enzymes into a mesophilic host, leading to an overproduction (de Carvalho 2011;2017).

Rhodococcus genus, belonging to the group Actinomycetes is able to use different organic compounds and pollutants as energy source and sole carbon, but also to produce diverse compounds and molecules with industrial interesting (Finnerty 1992; Bell, Philip *et al.* 1998). This metabolic diversity and versatility of *Rhodococcus* is related with the presence and mobilization of a large linear plasmids and with the presence of multiple homologues enzymes in catabolic pathways (de Carvalho and da Fonseca 2005b). Besides that, Rhodococci have been isolated from different sources such as soils, coastal sediments, rocks herbivorous dung, boreholes and groundwater (Bell, Philip *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). Thus, these characteristics may allow *Rhodococcus* to adapt and live in extreme conditions.

Environmental heterogeneity is an evolutionary challenge for organisms and therefore they need adaptation mechanisms to survive in extreme conditions, using one or more mechanisms depending the conditions. Thus, the study of the influence of other environmental conditions on the growth of these organisms 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. Besides that, the accumulation of storage lipids, characteristic in *Rhodococcus*, have an important role in survival under some environmental conditions (Hernández, M.A., Mohn *et al.* 2008).

The aim of this study was understanding the role of the lipids in the tolerance of *R. erythropolis* cells to dehydration, studying the effect of growth conditions on that and evaluate the role of storage lipids in cell tolerance to dehydration and antibiotic exposure.

2. Material and Methods

2.1. Bacterial Strain

R. erythropolis DCL14 was 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), Instituo 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 cells were grown in 100 mL Erlenmeyer flasks closed with rubber bungs, containing 20 mL of (i) mineral medium (MM) without or with salt (35 g/L NaCl, Carl Roth) supplemented with 0.25% (v/v) of absolute ethanol (Panreac) as carbon source or (ii) Mueller Hinton Broth (MH, Fluka Analytical). The composition of the mineral medium used is described by Cortes *et.al.* (Cortes and de Carvalho 2015).

The cultures were incubated at 30ºC and 200 rpm on incubator Agitorb 200 (Aralab). Cell growth was monitored by optical 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 biofilms cells

R. erythropolis DCL14 cells were grown on 24-well plates containing 1 mL of cell suspension with an initial OD600 of *ca.* 0.5-0.8. The cells, in this assay, were cultured in MM without and with salt supplemented with 0.25% (v/v) of absolute ethanol as carbon source and the plates were incubated at 30ºC 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 tubes (Eppendorf, Hamburg, Germany) and centrifuged (µSpeedFuge SFA13K Microcentrifuge, Savant Technologies) at 10,00 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 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 1.5 mL eppendorf tube, and the procedure described above for planktonic cells was followed.

The RapidVap evaporator was programmed at a temperature of 30ºC, without agitation, and a vacuum of 70 mbar. To assess the effect of desiccation in the *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 24h 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 fatty acid (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 with 1 mL of cell suspension was centrifuged 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, 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 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, 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 through MIDI calibration standards. The FAs were grouped into classes, to better understand the differences in the lipids 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 fatty acids (FA that do not belong to the previously mentioned classes). The degree of saturation (Dsat) of the fatty acids of the phospholipids of the cell membrane was defined as the ratio between the total SSFAs total and MUFAs.

2.6. Accumulation of storage lipids

Accumulation of storage of lipids in droplets and the production of exopolymeric glycolipids were promoted 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, three samples of 1 mL of each culture was collected with the objective to determining: (i) the tolerance of the cells

containing the lipid droplets to dehydration; (ii) the tolerance of these cells to vancomycin present in different concentration; 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.

2.6.1. Tolerance of the cells to antibiotics

2.6.1.1. 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 30ºC during 16-18h and the MIC was determined by observation of the lowest concentration that inhibited cell growth.

2.6.1.2. 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 30ºC 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 3500 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 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 plates. The plates were then incubated at 30ºC and the colony-forming units (CFUs) were counted after *ca*. 16-18h. To confirm if the cell were dead or required longer time for growth, the plates were kept at 30º C for up 48 h.

3. Results and Discussion

3.1. Dehydrations assays

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 determine. 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 of the cells along the time (data not shown). This also allowed the validation of the method used.

The results obtained in all wet weight sample analysis showed that the amount of water present in the *R. erythropolis* samples decreased with dehydration time, reaching a percentage between 23 and 30% of dry weight of the cells after 180 min in the evaporator, depending on the cell growth phase at the moment of harvest and the composition of growth media (data not shown). 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 (data not shown).

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 a cell cultivation in planktonic state. This planktonic growth was promoted in two different liquid broth and analysed in different stage of cell growth: at exponential and stationary phases. The planktonic cultures were dehydrated for different periods and the FAs of the cells were analysed (**Figure 1**). 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 1**).

Through the analysis of **Figure 1** it is possible to observe that the FA composition of cells is dependent of the growth medium and the 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* DCL14, 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 the age of the cells.

Analysing the exponential phase in MH (**Figure 1A**), 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 (data not shown). 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 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 cells stopped producing BCFA after 20 min of dehydration (from 1.3 ± 0.1% to 0%).

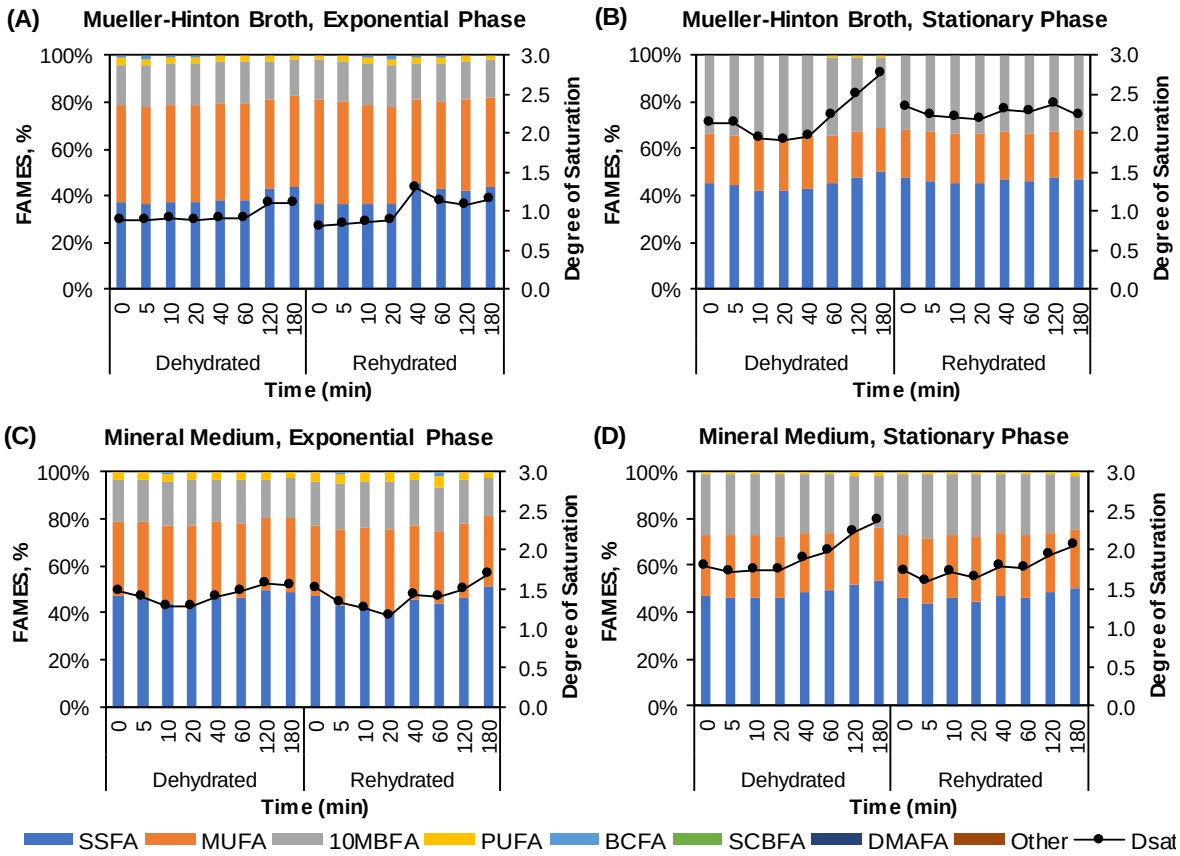

Figure 1 – Effect of growth media and dehydration time on the FA composition of *R. erythropolis* DCL14 cultures collected during different cell growth phases. The values represented are the mean of two independent assays. The line shows the corresponding degree of saturation of the FAs, and only those presented a concentration higher than 1% are shown**.**

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 1B**), only a slight decrease in the degree of saturation from 5 to 10 min and a significant increase after 20 min in the evaporator in the dehydrated cells were observed. Concomitantly to this increase in the percentage of SSFAs (from 43.6 ± 1.1% to 47.8 ± 2.2) the cells started producing PUFAs. 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.

Analysing the lipid composition of the cells grown in MM and collected during the exponential phase, it is possible observe a decrease followed by an increase in the degree of saturation due to the conversion of SSFAs into MUFAs or vice versa (**Figure 1C**). Besides the conversion in MUFAs, the SSFAs were also converted into 10MBFAs, as happened at 20 min of dehydration. For the remaining classes no significant alterations were observed. Regarding the rehydrated cells, a similar degree of saturation of FAs was observed (**Figure 1C)**. Lastly, analysing the lipid composition of the cells grown in MM and collected in the stationary phase, it is possible 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 control cells (**Figure 1D**).

In dehydrated cells, the stationary phase 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 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, significative 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 the cells grown on the two growth media. However, the cells 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 1**). 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).

3.1.2. 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 2**). 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 60 min (**Figure 2**).

Through the analysis of **Figure 2** 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 2**).

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.

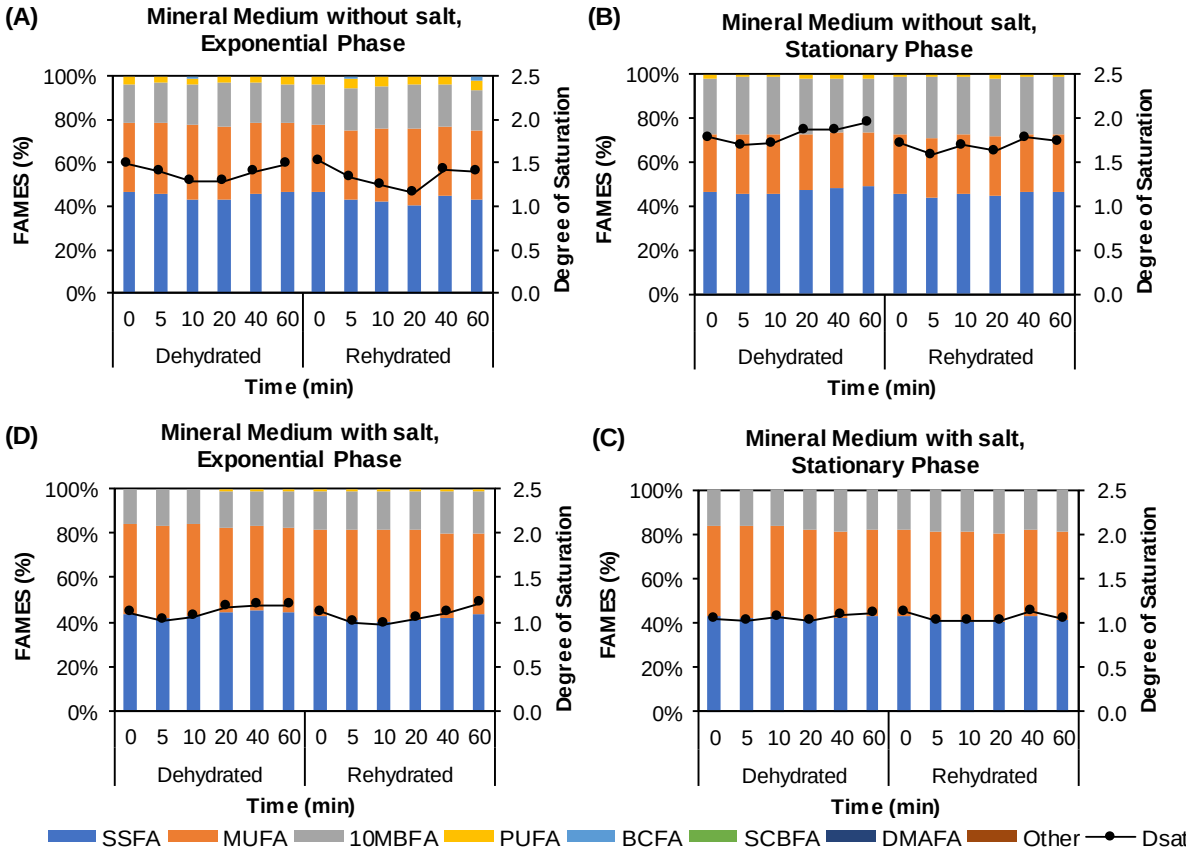

Figure 2 – Effect of salt concentration on growth media and dehydration time on FA composition of *R. erythropolis* DCL14 cultures collected during different cell growth phases. The values represented are the mean of two independent assays. The line shows the corresponding degree of saturation, and only the FAs with a concentration higher than 1% are shown**.**

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 2A**). 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 2B**) (for a more detailed analysis see section 3.1.1).

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 2C**), 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 due to a conversion of MUFAs in SSFAs but also to a conversion in PUFAs. 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 2D**).

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 amount of water (about 95%) that are incorporated into its structure by hydrogen bonding and how dries slower than its surroundings, because their hygroscopic nature, 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 3**). 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 60 min (**Figure 3**).

Through the analysis of **Figure 3**, 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 3**). 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 3B**). 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 due a conversion of SSFAs in MUFAs. 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 3B**). 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%). 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 3D**). 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.

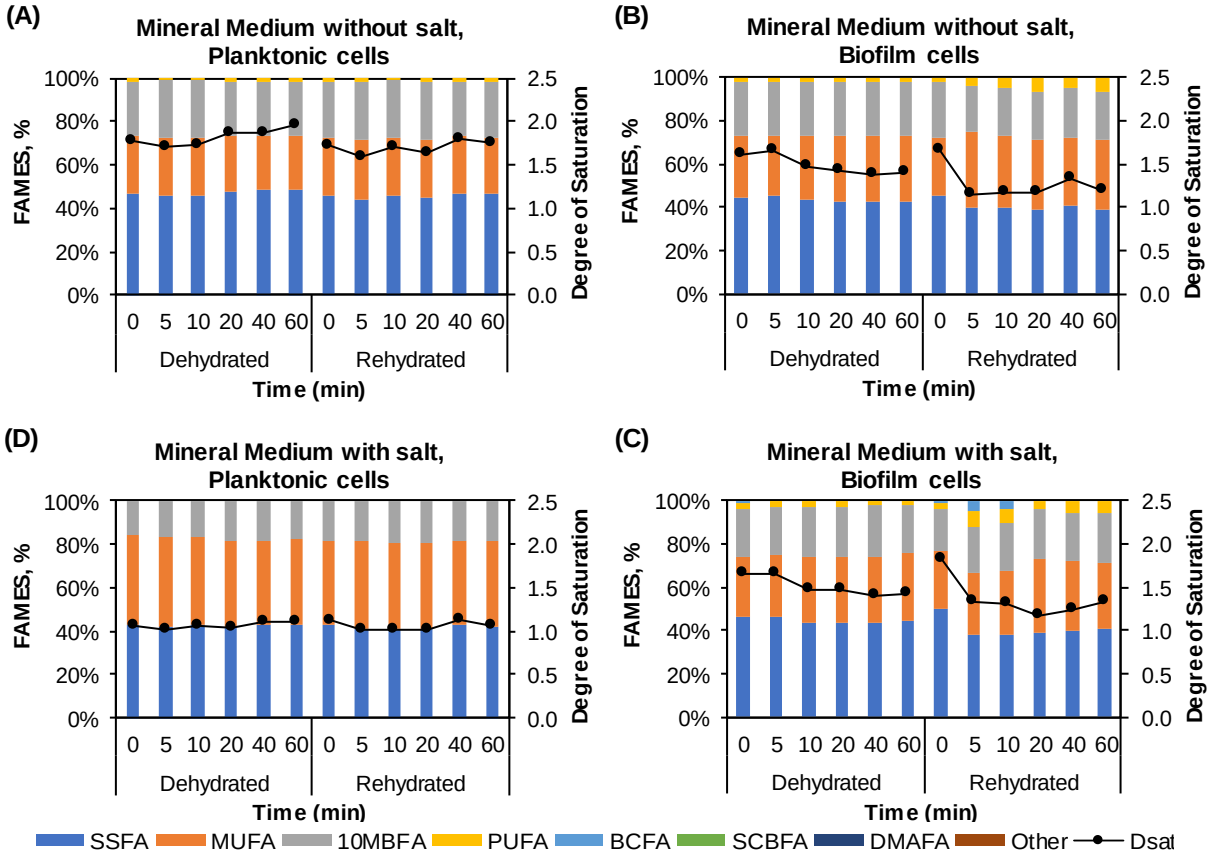

Figure 3 – Effect of dehydration time on FA composition of *R. erythropolis* DCL14 cultures grown in different media in planktonic and biofilm modes. The values represented are the mean of two independent assays. The line shows the corresponding degree of saturation, and only the FAs with a concentration higher than 1% are shown**.**

3.2. Influence of sugar concentration on cell adaptation

Cell cultures in late stationary phase are known to be more resistance to stressful conditions (Kolter, Siegele *et al.* 1993; 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 (data not shown) and secondly under stress conditions (**Figure 4**). Under non-stress conditions, it was verified that both sugars and their concentrations did not influence the FA profile of *R. erythropolis* DCL14 (data not shown). Thus, was also relevant to study their influence under stress conditions (**Figure 4**).

Analysing **Figure 4,** 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.).

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.

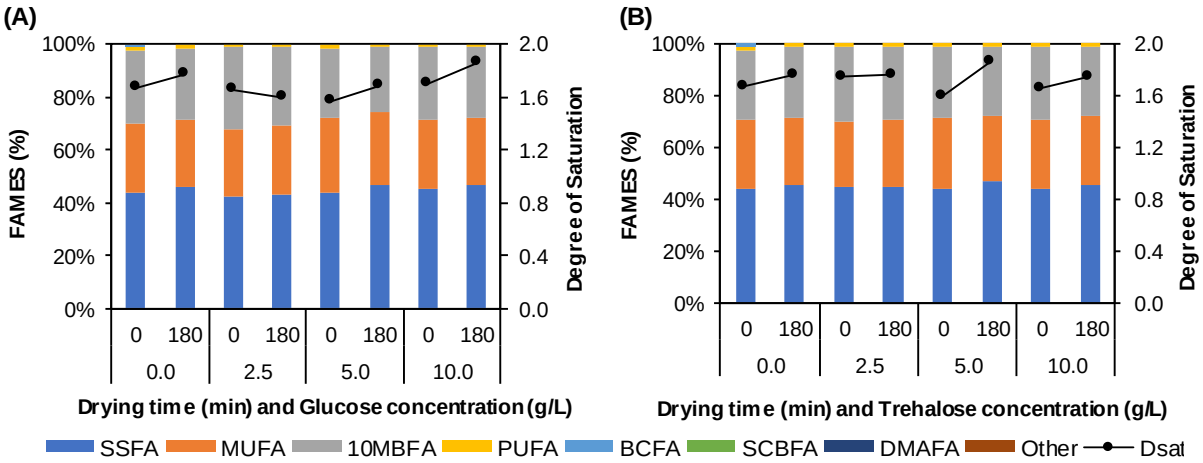

Figure 4 – Effect of the type sugar and concentration on dehydration-resistance at the level of the composition of the fatty acid, on *R. erythropolis* DCL14 cultures. The values represented are the mean of two independent assays. The line shows the corresponding degree of saturation, and only the FAs with a concentration higher than 1% are shown**.**

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).

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.

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. Through the analysis of the MIC values obtained, a concentration of vancomycin of 2 g / L was chosen for the following analyses (data not shown).

3.2.2.1. Time-Dependent Killing

In **Figure 5** it was shown the susceptibility of exponential growing *R. erythropolis* DCL14 cultures to vancomycin, 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 5**).

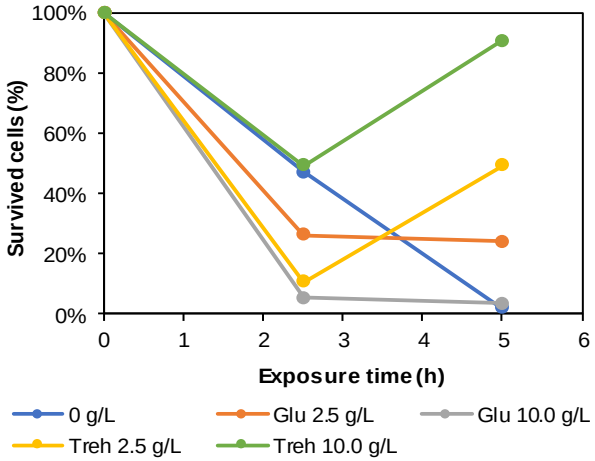

Figure 5 – Percentage of *R. erythropolis* DCL14 that survive during 5 h of exposure to 2 µg/mL of vancomycin in different concentration of glucose (Glu) and trehalose (Treh) as carbon source**.**

It is possible to observe in figure 5, 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).

3.2.2.1. 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 5**) and it was analysed the variations at the level of the composition of the FA (**Figure 6**).

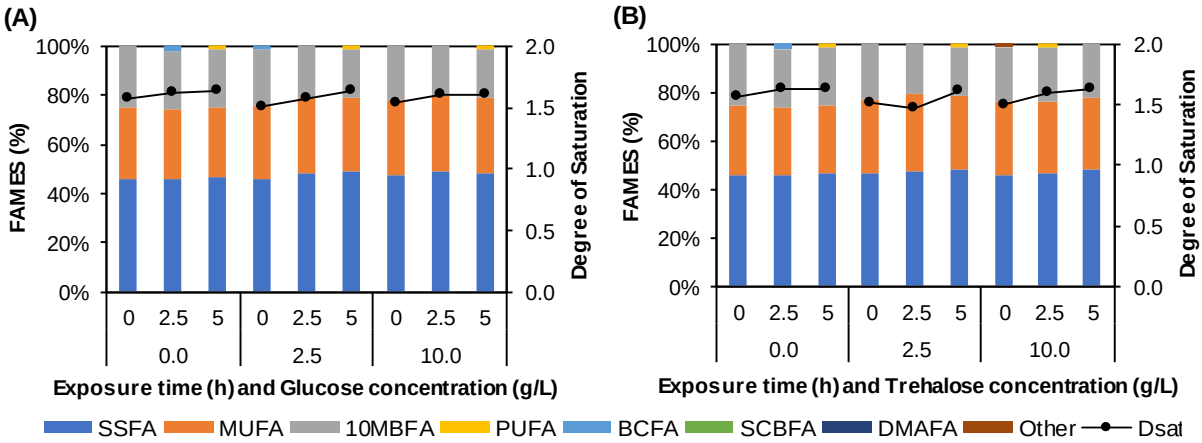

Figure 6 – Effect of the type of sugar and concentration on antibiotic-resistance during the time of exposure at the level of the composition of the fatty acids, on *R. erythropolis* DCL14 cultures. The line shows the corresponding degree of saturation, and only the FAs with a concentration higher than 1% are shown**.**

Through analysis of **Figure 6**, 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

When exposed to dehydration, *Rhodococcus* cells changed their FA composition in order to achieve the best membrane fluidity that allowed the survival. For the cells grown in planktonic state, for the three media used, were observed an increase in the degree of saturation of the membrane, which indicates that the cell adjusted their membrane composition to reduce the fluidity of the cellular membrane. This reduction in fluidity avoided the leakage of intracellular compounds. Comparing to what was observed for the planktonic cells, biofilm cells decreased the degree of saturation, which resulted in a more fluid membrane.

The promoted lipid accumulation aimed at assessing if 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. 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.

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